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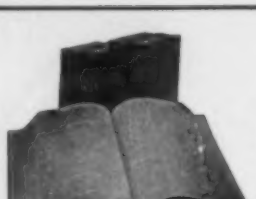

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*Scientific Edition*

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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# The Colorimetric Determination of Lidocaine With *cis*-Aconitic Anhydride*†

By EDWARD G. FELDMANN and HENRY M. KOEHLER

**The quantitative reaction between tertiary amines and *cis*-aconitic anhydride has been applied to the assay of microquantities of the local anesthetic lidocaine. The modified colorimetric method which is employed provides a particularly useful selective means for the assay of small samples containing this drug, which might arise from dosage form studies or biological investigations. The procedure detailed is quite simple in operation but yet shows relatively high precision and accuracy over the suggested range.**

THE ANALYSIS of microquantities of the local anesthetic lidocaine (α-diethylamino-2,6-acetoxylidide) and its salts has long presented a problem to research workers. Most methods which are presently available, such as acid-base titrations (1, 2), potentiometric titration (3), and quantitative nitration (4), require relatively large samples and are not suited to microanalysis. Two procedures which have been employed as micromethods involve precipitation with reinecke salts (5, 6) or complex formation with methyl orange (7). Both of these methods however, suffer in that they are not too specific and practically any other amino compound present will give rise to a very significant degree of interference.

Moreover, there are other limitations to each of these methods which further restricts their usefulness. Reinecke salts often do not give quantitative precipitation, and in addition, relatively large quantities of the precipitate may be lost mechanically during the washing process, or significant amounts of excess reagent adhering to the precipitate may be carried over due to incomplete washing. In the assay with methyl orange some of the sample may be lost during the step in which the ethylene dichloride sample solution is washed. This is particularly a problem if the pH is not adequately controlled. Furthermore, as Brodie and co-workers (8) have pointed out, "The sensitivity of the reaction in this solvent (ethylene dichloride) is limited by some solubility of free methyl orange in ethylene dichloride. . . ." This would, of course, give rise to some additional error.

In spite of the limitations listed, each of these procedures is useful in certain instances. Even in these cases, however, particularly because of the questionable specificity of these methods, it would be highly advantageous to have another analytical procedure available—particularly one which might be more selective.

A simple, selective micromethod for the determination of lidocaine would be useful both in drug assay work, where it often is necessary to analyze less than a single dosage unit[1] (1 to 2 ml. of a 1 to 2 per cent solution) of the anesthetic agent, or in the determination of the quantity of lidocaine present in small quantities of biological materials used in connection with pharmacological studies of the drug. Simple ultraviolet spectroscopy, which has proved to be extremely useful in such cases when most other local anesthetic compounds are considered, is of limited value in the case of lidocaine because of its significantly lower absorption in this spectral range. The absorptivity of

* Received May 6, 1959, from the Division of Chemistry, American Dental Association, Chicago 11, Ill.

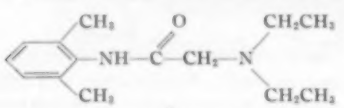
† Analysis of Local Anesthetics III. For the preceding paper in this series see THIS JOURNAL, **48**, 197(1959).

The authors wish to express their appreciation to Dr. J. Roy Doty for his helpful comments, and to Mrs. Helen Jones for her assistance in preparing the manuscript.

[1] Astra Pharmaceutical Products, Inc., markets solutions for injection containing lidocaine hydrochloride under the brand name of Xylocaine hydrochloride.

an aqueous solution of lidocaine hydrochloride at its maximum wavelength, for example, is less than 5 per cent of that observed with procaine hydrochloride, butethamine hydrochloride, naepaine hydrochloride, or tetracaine hydrochloride at their respective wavelengths of maximum absorption. As a result, it is rather difficult to assay small quantities of lidocaine by ultraviolet spectroscopy; even in those cases where larger samples or greater concentrations of the drug are available, one must expect larger errors since impurities displaying absorption at the same wavelength give rise to a relatively greater degree of interference. This follows because it is not possible to reduce the effect of such impurities through dilution to the same extent as in the case of the other anesthetic compounds cited. For these same reasons, spectrophotofluorometry has also been of limited practical value (9).

Lidocaine is practically unique among the commonly used local anesthetic agents in that it does not contain either a primary or secondary amino group. Inspection of the structural formula for this compound, which is illustrated (I), shows that



I

chemically the only functional groups are an amide and a tertiary amine. Since amides historically do not lend themselves to microanalysis, a method of analysis based upon the tertiary amine was sought. Inspection of the literature shows that few methods, other than those which have been noted above as having been employed for lidocaine, are available for the determination of tertiary amines. Furthermore, these include no assay procedure of a truly micro nature.

Very recently, however, Sass and co-workers (10) reported a general method for the determination of tertiary aliphatic amines employing a reaction first described by Palumbo (11) as a qualitative procedure, and later extended by Cromwell (12) to the quantitative determination of trimethylamine in biological samples. This procedure appeared to hold promise of being potentially useful in the analysis of lidocaine. Subsequent experimental investigation in this laboratory showed that upon making several relatively minor modifications, a generally useful method for the determination of micro quantities of the anesthetic agent resulted. It should be noted, however, that in the assay of lidocaine contained in either dosage form samples or biological materials, the presence of other tertiary amines, which might be carried over in the toluene extraction, will give rise to interference when using this procedure.

## EXPERIMENTAL

**Chemicals.**—Samples of lidocaine free base were generously supplied by Astra Pharmaceutical Products, Inc. *cis*-Aconitic anhydride was purchased from several chemical manufacturers. Melting points of the various samples and the colorimetric response of reagents prepared from these samples differed considerably, however. Uniformly good results with negligible blank readings were regularly obtained using samples purchased from the California Corporation for Biochemical Research. Reagent grade acetic anhydride (J. T. Baker) was routinely redistilled and only the fraction boiling at 138–140° was collected. Toluene, employed for the preparation of the reagent and also for dilution of standards and samples, was routinely purified because even reagent grade toluene, when used directly, gave rise to variable blank readings. The following purification method was found to yield toluene of uniformly high purity.

Five liters of reagent grade toluene (Merck) is shaken for several minutes in a large separatory funnel with about 300 ml. of concentrated sulfuric acid. The acid layer is withdrawn and the toluene is washed with two portions of water (200–300 ml.). The organic layer is transferred to a large reagent bottle, and a quantity (200–300 Gm.) of anhydrous sodium sulfate is introduced; the bottle is stoppered and shaken periodically over several hours. The toluene is then filtered and distilled; the fraction boiling at 109–111° is collected.

**Reagent.**—An accurately weighed 0.25-Gm. portion of *cis*-aconitic anhydride is transferred to a 100-ml. volumetric flask, and 40 ml. of redistilled acetic anhydride is introduced. The flask and its contents are swirled to effect complete solution; the flask may be warmed gently if necessary, and then cooled. The solution is diluted to volume with purified toluene and mixed. The solution is stored in a glass-stoppered bottle and allowed to age at room temperature for twenty-four hours prior to use. Under conditions of low humidity the solution appears to be stable for about two weeks.

**Apparatus.**—All glassware employed must be scrupulously clean and particularly free of contamination by any material which can act as a Lewis base. It should be noted that this includes certain types of detergents. A Beckman Model DK-2 recording spectrophotometer, with silica cells of 1-cm. light path, was employed to record the absorption spectra. The same instrument, with a model 92430 time drive attachment, was used in the color stability study. Absorbance values at peak wavelengths were checked, employing a Beckman DU spectrophotometer. All other absorption measurements, including those taken for the preparation of standard curves, were made using a Klett-Summerson colorimeter with a No. 54 filter.

**Procedure.**—*Preparation of Standard and Sample Solutions.*—An accurately weighed 120-mg. sample of pure lidocaine base is placed in a 100-ml. volumetric flask, the flask is filled to the mark with toluene, and mixed. A 5.0-ml. aliquot of this solution is transferred to a 50-ml. volumetric flask, diluted to volume with toluene, and mixed (standard solution A).

A 25-ml. aliquot of solution A is similarly diluted to 50 ml. (standard solution B), and a 10-ml. aliquot of solution B is diluted to 50 ml. (standard solution C). This dilution scheme provides standard solutions covering the upper limit (standard A; 120 μg. per ml.), the middle (standard B; 60 μg. per ml.), and the lower limit (standard C; 12 μg. per ml.) of the assay range combined with a conservative use of purified toluene and reliability in the accuracy of dilutions.

An accurately measured aqueous or aqueous acid solution of the unknown lidocaine sample is made alkaline in a separatory funnel with ammonia T. S. and is promptly extracted with an accurately measured volume of toluene. The bulk of the toluene layer is removed, centrifuged briefly, and an aliquot is withdrawn for dilution to the desired concentration so that readings will fall within the useable range.

*Color Development.*—Samples of 2.0 ml. each of the standard and unknown solutions are placed in 10-ml. glass-stoppered volumetric flasks. A 2.0-ml. sample of toluene is placed in another flask to serve as a reagent blank. To each flask is added 1 ml. of the *cis*-aconitic anhydride reagent, the contents are mixed, and the flasks are loosely stoppered. Each flask is heated in a boiling water bath for forty-five seconds and allowed to stand at room temperature for fifteen minutes. The solutions are then diluted to volume with a solution containing 20% acetic anhydride and 80% toluene (v/v), mixed, and allowed to stand at room temperature for an additional fifteen minutes.

Readings of color intensity are taken with the instrument set using toluene as the reference solvent. The readings obtained with standard samples are graphed and the concentration of lidocaine in the unknown sample is determined from the resulting standard curve.

## RESULTS AND DISCUSSION

**Extraction into Toluene.**—Analysis of aqueous solutions of lidocaine hydrochloride are dependent upon the completeness with which the lidocaine free base in such solutions is extracted with a single portion of toluene. The completeness of extraction was checked by two methods. In the first, a sample of standard lidocaine base was made up in aqueous hydrochloric acid and the color developed and measured after extraction, while another sample was dissolved directly in toluene and the color was developed and measured. The readings for equal concentrations were the same in each case, indicating that the entire sample of lidocaine carried on through. The second method employed was a variation of the N. F. method (1) in which an aliquot of a single toluene extract was taken rather than pooling several chloroform extracts. From the titration results it was again demonstrated that complete carry-over into toluene occurs with a single such extraction.

**Reaction Involved.**—Sass (10) has postulated that the reaction proceeds between *cis*-aconitic anhydride and a tertiary amine to form a complex ionic species. The structure of the postulated product is such that it would be expected to present a number of resonance forms which serves to explain the highly colored nature of the complex. The absorption curve in the visible region obtained with a typical sample of the colored lidocaine complex is shown in Fig. 1. It can be seen from the figure that the absorption maximum occurs at about 540 mμ. It should also be noted that the absorption of a reagent blank, similarly run, is very low at this wavelength. Both of these absorption curves were taken against toluene as a reference solvent.

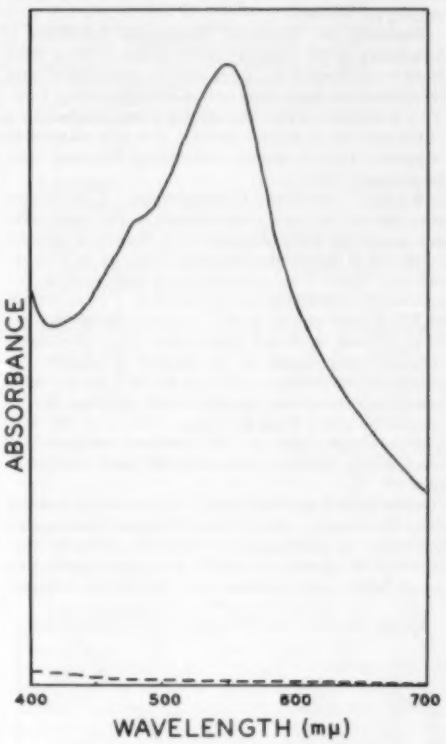



Fig. 1.—Visible absorbance curves of color complex; —— lidocaine complex; – – – – reagent blank.

**Dilution with Acetic Anhydride-Toluene.**—In some cases in which relatively large concentrations of anesthetic were employed, some precipitation of the colored lidocaine complex of *cis*-aconitic anhydride was noted if final sample dilution was made simply with toluene, as in the procedure suggested by Sass. It was found, however, that use of a mixed solvent containing 20% acetic anhydride prevented precipitation even at anesthetic concentrations exceeding the useful range of determination. Consequently, the use of this mixed solvent is recommended routinely in the procedure for purposes of final dilution after color development.

**Optimum Heating Period.**—A series of lidocaine solutions containing the *cis*-aconitic anhydride reagent were heated for various time intervals to establish the optimum length of heating. It was found that the most consistent, reproducible readings resulted when solutions were heated for more than thirty seconds and less than sixty seconds. As a result, forty-five seconds is recommended.

**Stability of the Reagent.**—As noted above, the reagent must be aged for twenty-four hours prior to use. Reproducible readings were obtained with *cis*-aconitic anhydride solutions which were two weeks old; after two weeks readings tend to become gradually lower. Since standard solutions are run simultaneously with unknown samples, this is not a critical consideration but it is not recommended that reagent solutions older than two weeks be employed routinely.

**Stability of Standard Lidocaine Solutions.**—Gradually lower readings were obtained even with fresh reagent when the same toluene solutions of pure lidocaine base were used over a period of some time. This would appear to be due to gradual degradation of the anesthetic in the solvent. For this reason it is suggested that standard solutions of lidocaine base be prepared daily.

**Range of Anesthetic Concentration.**—Colorimeter readings which were proportional to the anesthetic concentration were obtained with standard toluene solutions of lidocaine containing from 12 to 120 μg. per ml. Since 2 ml. of solution is employed in the procedure this would be equivalent to a total sample of $1.0 \times 10^{-6}$ to $1.0 \times 10^{-7}$ moles. At concentrations higher or lower than this range the color response was found to be grossly nonlinear. A graph of colorimeter readings plotted *versus* lidocaine concentrations is illustrated in Fig. 2. It should be noted from the figure that over the suggested range there is an excellent straight-line relationship between color intensity and concentration.

**Stability of Color Produced.**—After addition of the color developing reagent and subsequent heating, an initial fifteen-minute period of standing at room temperature is allowed to enable complete reaction to occur before the solutions are diluted to volume.

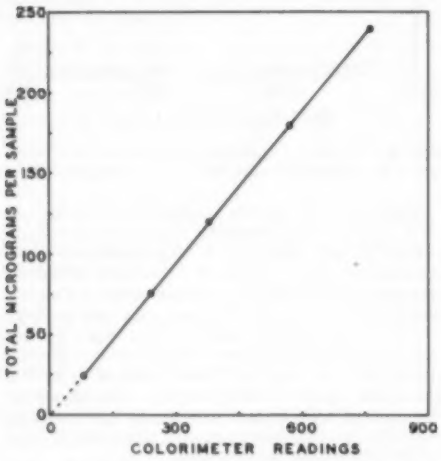



Fig. 2.—Typical standard curve prepared by plotting lidocaine base concentrations *versus* colorimeter readings obtained after color development.

Experimentally, color intensities of several test samples were then taken over a two-hour interval using a recording spectrophotometer. It was noted that the color intensity drops rather sharply during the first few minutes, levels off for some five to fifteen minutes, and then continues to fall off very gradually for the balance of the time studied. These results indicated that readings are most reproducible when taken fifteen minutes after the solutions have been diluted to volume.

**Precision and Accuracy.**—Some indication of the precision or reproducibility of the method can be seen by examination of Table I.

TABLE I.—COLORIMETER READINGS OF REPLICATE LIDOCAINE SAMPLES[a]

| Sample | Reading | Sample | Reading |
|---|---|---|---|
| 1 | 378 | 5 | 381 |
| 2 | 375 | 6 | 384 |
| 3 | 370 | 7 | 378 |
| 4 | 380 | 8 | 376 |

Mean—377.8. Standard deviation—4.2. Precision—1.1%.

[a] Each sample contained a total of 120 μg. lidocaine base.

Eight replicates of a standard lidocaine solution were treated by the procedure described and the colorimeter readings obtained with the resulting solutions are given in the table.

A stock solution of aqueous "synthetic commercial solution," prepared with accurately weighed portions of each ingredient, in the same concentrations as listed by the manufacturer's label, was made up as a standard solution which would simulate the commercial product. Portions of this solution in varying volumes, some of which were as small as 1 ml., were then run through the toluene extraction, and after appropriate dilution were subjected to color development.

The data from these experiments indicated that an accuracy of ± 2–3% can be anticipated from the method when the procedure is followed carefully and the cautions detailed herein, such as purity of solvents and glassware cleanliness, are rigidly followed.

## REFERENCES

(1) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 326.
(2) Bullock, K., and Grundy, J., *J. Pharm. and Pharmacol.*, **7**, 755(1955).
(3) Ceglie, A., and Malatesta, P., *Farmaco Pavia, Ed. sci.*, **9**, 598(1954).
(4) Örtenblad, B., *Acta Chem. Scand.*, **5**, 672(1951).
(5) Örtenblad, B., and Jonsson, K., *ibid.*, **5**, 510(1951).
(6) Hanquin, J., and Lapiere, C., *J. pharm. Belg.*, **10**, 246(1955).
(7) Sung, C., and Truant, A., *J. Pharmacol. Exptl. Therap.*, **112**, 432(1954).
(8) Brodie, B., Udenfriend, S., and Dill, W., *J. Biol. Chem.*, **168**, 335(1947).
(9) Personal communication from personnel at the National Heart Institute, National Institutes of Health, Bethesda, Md.
(10) Sass, S., Kaufman, J., Cardenas, A., and Martin, J., *Anal. Chem.*, **29**, 529(1958).
(11) Palumbo, M., *Farm. sci. e tec. Pavia*, **3**, 675(1948).
(12) Cromwell, B., *Biochem. J.*, **46**, 578(1950).

# Determination of Epinephrine in the Presence of Degradation Products*

By TAKERU HIGUCHI, THEODORE D. SOKOLOSKI, and LOUIS C. SCHROETER

**The chemistry of the presently official method of analysis for epinephrine has been closely examined. Results show that the official procedure may lead to formation of other acetylation products in addition to the expected triacetylepinephrine (TAE). Evidence is also offered showing that some oxidation products are acetylated and extracted. A modification is proposed based on chromatographic isolation of TAE in place of the time-consuming extraction step and close control of the acetylation step. Results of a comparative study of four possible assay methods for epinephrine are also presented.**

DETERMINATION of epinephrine in the presence of its degradation products presents an unusually difficult analytical problem. Although the method of the U. S. P. XV (1) based on the work of Welsh (2) represents a major step[1] in solving the problem, it possesses certain unsatisfactory features. In the present work an attempt has been made to shorten and improve the official method. The basic method as proposed by Welsh consists of triacetylation of the drug, exhaustive extraction of the acetylated product with chloroform, and gravimetric determination of the extracted solids. The acetylation step was introduced to convert the unextractable, difficultly-soluble epinephrine base into the freely-soluble triacetyl form. Results of the present investigation show that in the official method the weighed product is usually not pure acetylated compound and this leads to variable errors. The present communication is concerned with modifications which appear to overcome the shortcomings of the official assay method.

## EXPERIMENTAL

The following experimental conditions were generally observed in carrying out these studies:

**Sample Preparation.**—Epinephrine solutions containing chlorobutanol were transferred to a separatory funnel and extracted four times with equal volumes of reagent grade carbon tetrachloride. Five milliliters of the aqueous solution, representing about 5 mg. of epinephrine base, was transferred to a 50-ml. tall beaker and a stirring magnet added. Bisulfite or sulfite, if present, was oxidized by microiodine titration to the starch end point—slight excess of iodine was destroyed by the addition of 0.01 *N* sodium thiosulfate. Twenty-five milliliters of reagent grade isooctane was added to the beaker.

**Acetylation.**—The $O^3$, $O^4$, N-triacetyl derivative of epinephrine (TAE) was quantitatively prepared by introducing a total of 0.23 ml. acetic anhydride in divided portions and 435 mg. sodium bicarbonate to the epinephrine solution with rapid stirring. A typical schedule is shown below.

| Acetic Anhydride, ml. | Sod. Bicarbonate, mg. | Stirring Time, sec. |
|---|---|---|
| 0.13 | 435 | 60 |
| 0.05 | ... | 60 |
| 0.05 | ... | 60 |
| Total 0.23 | 435 | 180 |

For studies made with varying time of reaction, approximately the same proportions of time was used for each step. About 100 mg. citric acid was added to buffer and stabilize the reaction mixture after completion of acetylation. The mixture was stirred while 5.6 Gm. of Celite 545[2] was added in portions to prepare a uniform slurry for chromatographic assay.

**Chromatography.**—Fourteen grams of Celite 545 wetted with 12.6 ml. of pH 4, 0.1 molar citrate buffer was slurried with isooctane and packed into a chromatographic column (2 × 45 cm.) in portions as described by Higuchi and Patel (3). Excess isooctane was drained from the column and the previously prepared slurried acetylated mixture was quantitatively transferred and packed into the prepared column. The reaction beaker was rinsed with a slurry of isooctane and 1 Gm. of Celite wetted with 0.9 ml. buffer; this was also packed into the column. The finished column was drained of isooctane and developed with chloroform. The first 10-ml. eluate was discarded, then exactly 50 ml. was collected for spectrophotometric or gravimetric determination. Actually several runs showed that essentially all of the TAE in the sample was contained between the 20th and 30th ml. of eluate, but additional volume was collected on either side of the peak to insure total recovery.

**Spectrophotometry and Polarimetry.**—The ultraviolet spectrum of the eluates was determined with a Cary recording or Beckman DU spectrophotometer. Solutions which exhibited low absorbances were concentrated from 50 ml., by evaporation, to 25 ml.

Determination of optical activity of the triacetyl derivative, when employed, was carried out by first evaporating combined eluates representing about 17 mg. of the derivative. The eluates were evaporated to dryness with gentle heat and dissolved in about

* Received December 29, 1958, from the School of Pharmacy, University of Wisconsin, Madison.
This project was in part conducted under a contract with Armed Forces Medical Procurement Agency, Brooklyn, N. Y., and was in part supported by a grant from Parke, Davis and Co., Detroit, Mich.

[1] The development of this general procedure by Welsh for epinephrine represents a very significant advance in the science of analytical chemistry. The theoretical rationale underlying the method probably has not been fully appreciated.

[2] Johns-Manville Corp.

0.5 ml. of chloroform. The chloroform solution was quantitatively transferred to a 1-dm. micro polarimeter tube of known volume (1.65 ml.) and the volume carefully adjusted with chloroform rinsings. Measurements were made at 25° with a Zeiss-Winkel polarimeter, using sodium D-line (589 mμ) light.

**Test for Stability of TAE Against Hydrolysis in the Internal Phase.**—Stability of the triacetyl derivative to hydrolysis when present in the internal phase was tested by dissolving triacetylepinephrine in pH 3.90 and pH 5.58, 0.1 molar, citrate buffers and storing the solutions at 25°. Aliquots of the solutions were slurried with Celite 545 and isooctane, as described above, and packed into prepared chromatographic columns. Columns were developed with chloroform. Extent of hydrolysis was determined by ultraviolet spectrophotometry of the eluate and gravimetric determination of its residue.

**Test for Recovery of TAE from Chromatographic Columns.**—Recoveries from columns buffered at pH 4 with 0.1 molar citrate was tested with $O^3$, $O^4$, N-triacetyl-*l*-epinephrine, m. p. 94–95°, $[\alpha]_D^{25}$ −94.5. Solutions of triacetylepinephrine were slurried with Celite 545 and isooctane and packed into chromatographic columns. The columns were developed with chloroform. Triacetylepinephrine was determined by ultraviolet spectrophotometry of the eluate and gravimetric determination of the residue.

**Comparative Study.**—Solutions containing epinephrine in the presence of degraded material were analyzed by ultraviolet spectrophotometry, a modified U. S. P. XIV colorimetric procedure (4), the U. S. P. XV acetylation procedure, and by this modified acetylation procedure. Epinephrine solution was degraded by oxidation in a sealed system in which the oxygen atmosphere was maintained at 1.5 atmospheres for one hundred hours at 80°. Analysis of the resulting black, insoluble, degraded product by the acetylation procedure indicated no epinephrine present. The degraded product along with synthetic *l*-epinephrine was used to prepare known test solutions.

The solutions were buffered at pH 4.0 with 0.1 molar acetate, allowed to stand four hours, then filtered several times to remove all insoluble material. Ultraviolet spectra of the solutions, diluted to equal 1-Gm. epinephrine base in 11,200 ml., were recorded with a Cary recording spectrophotometer from 250 to 310 mμ. The absorbance at the 280 mμ maximum was compared with a Beer's law plot.

Solutions were also analyzed by a modified U. S. P. XIV (4) ferro-citrate colorimetric procedure. Five milliliters of the solution was diluted to 25 ml. with 1:500 sodium bisulfite solution; a 5-ml. aliquot was transferred to a 25-ml. volumetric flask containing 10 ml. of 1:500 sodium bisulfite solution. To the solution was added 0.5 ml. of ferro-citrate and 5 ml. of buffer solution prepared as described in the U. S. P. XIV. The volume was adjusted to 25.0 ml. and absorbance determined at 530 mμ in a Bausch and Lomb model 20 colorimeter after thirty minutes color development. Absorbances were compared with a Beer's law plot and the results tabulated.

The U. S. P. XV assay and the modified acetylation procedure described in the prior section were employed in comparative analyses of the solutions. The solutions were diluted for the official assay with equal volumes of pH 4, 0.1 molar acetate buffer. Thirty milliliters of diluted solution was acetylated and the weight of chloroform-soluble acetylated product determined. Ultraviolet spectra of those amorphous residues which gave gravimetric recoveries in excess of 100% were determined in 50 ml. chloroform solution. Optical rotation of 5 ml. chloroform solutions of the residues was determined with a Zeiss-Winkel polarimeter at 25° with sodium D line light, using a 1-dm. micro tube (Table IV). Analytical values obtained with the U. S. P. and the modified procedure refer to *l*-epinephrine determined at $O^3$, $O^4$, N-triacetyl-*l*-epinephrine.

## RESULTS AND DISCUSSION

Experimental evidence obtained from these studies strongly indicates that the method of assay for epinephrine as described in the U. S. P. XV is based partially on uncertain assumptions. Close analysis of the reaction system does not point to straightforward quantitative formation of triacetylepinephrine from the catecholamine under the official procedure. Prolonged reaction of the drug with acetic anhydride apparently leads to production of other reaction products in addition to the major product, TAE. This tendency not only interferes with the gravimetric determination of the acetylated material but also with analytical interpretations based on the optical activity of the isolated reaction products.

Formation of another product at the expense of TAE is suggested by results of chromatographic analysis of the acetylation product. When the acetylated reaction product, subjected to varying reaction times, was analyzed for its triacetyl content on a column, the results shown in Fig. 1 were obtained. The TAE shown on the figure (marked spectrophotometric) were determined spectrophotometrically following chromatographic isolation. The data suggest that quantitative conversion of epinephrine to the triacetyl (TAE) derivative appears to occur within the first two minutes. Longer reaction time seems to favor further reaction to give a significant amount of uncharacterized acetylation product, hereafter referred to as peracetyl compound. Decrease in actual yield of TAE with continued reaction was not attributable to hydrolysis of the formed derivative since concurrent gravimetric determinations on the same eluates showed approximately 100% apparent recovery of chloroform-soluble product calculated as TAE, as also shown in Fig. 1.

It is apparent from Fig. 1, that the official gravimetric method yields fairly acceptable results primarily because the other product seems to have similar solubility characteristics and is extracted along with TAE. It may be concluded, nevertheless, that a prolonged reaction period leading to formation of an end product other than TAE will tend to give high values in the official procedure.

There existed a possibility that the apparent low recovery of TAE may be partly attributable to possible hydrolysis of the product during its chromatographic isolation. Experimental tests indicate that this is not the case, as shown in Table I. The triacetyl derivative was found to be stable towards

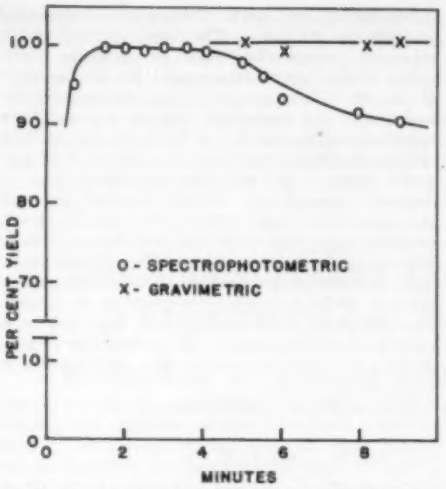



Fig. 1.—Apparent yield of triacetylepinephrine as function of reaction time. The circles represent results obtained by spectrophotometric determination on chromatographically isolated TAE. The crosses are results of direct gravimetric determination on total chloroform extracts.

hydrolysis for at least twenty-four hours when dissolved in pH 3.9–5.6 citrate buffers. Results of a direct test of recovery of triacetylepinephrine from chromatographic columns buffered at pH 4 with 0.1 molar citrate buffer are shown in Table II. The citrate buffered internal phase was found to give good separation of the acetylation mixture, triacetylepinephrine being quantitatively eluted and degraded products being retained.

Direct extraction of products of acetylation may lead to recovery of not only triacetylepinephrine but also certain acetylated forms of oxidized epinephrine as well as the reaction products which seem to result from exhaustive acetylation. The difficulty in reproducing the same degree of acetylation and extraction is evident in Fig. 2. The two upper spectral

TABLE I.—STABILITY OF O³, O⁴, N-TRIACETYL-*levo*-EPINEPHRINE IN 0.1 MOLAR CITRATE BUFFER SOLUTIONS AT 25°

| pH | Residence Time, Hr. | Added, mg. | Found,[a] mg. | Recovery, % |
|---|---|---|---|---|
| 3.90 | 0.25 | 76.1 | 76.3 | 100.3 |
| 3.90 | 24.0 | 76.1 | 78.3 | 102.8 |
| 5.58 | 0.25 | 68.7 | 69.1 | 100.5 |
| 5.58 | 24.0 | 68.7 | 69.7 | 101.5 |

[a] Determined gravimetrically following chromatographic isolation.

TABLE II.—RECOVERY OF O³, O⁴, N-TRIACETYL-*levo*-EPINEPHRINE FROM CHROMATOGRAPHIC COLUMNS[a]

| Column No. | Added, mg. | Found,[b] mg. | Recovery, % |
|---|---|---|---|
| 1 | 17.3 | 17.6 | 101.7 |
| 2 | 17.3 | 17.2 | 99.4 |
| 3 | 12.6 | 12.4 | 98.4 |
| 4 | 14.9 | 14.9 | 100.0 |

[a] Internal phase buffered at pH 4 with 0.1 molar citrate.
[b] Determined gravimetrically.

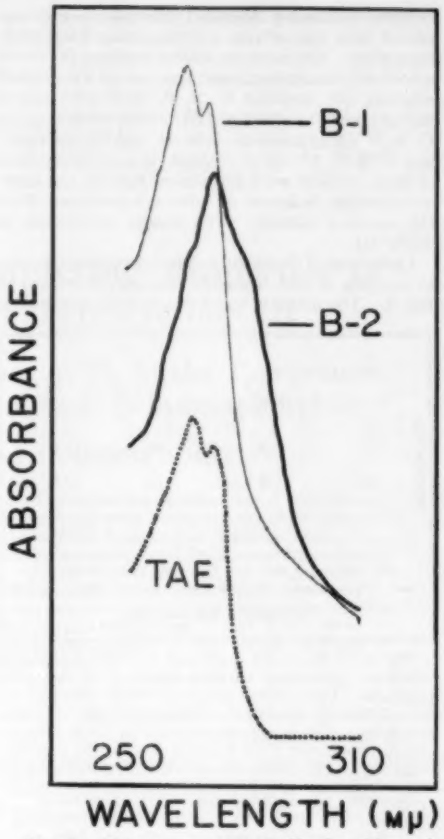



Fig. 2.—UV Spectra (B-1, B-2) of residues obtained in duplicate analyses of solution B (0.10% epinephrine, 0.10% degraded material) by U. S. P. XV procedure. Lower curve (TAE) is the ultraviolet spectrum of O³, O⁴, N-triacetyl-l-epinephrine isolated by chromatography of residues.

curves were obtained on extracts from duplicate analyses following the official procedure on a sample containing 0.1% epinephrine and 0.1% degraded epinephrine. The lower curve was found following chromatographic isolation of TAE from these runs.

The probable presence of acetylation product(s?) other than TAE, which tends to give high results gravimetrically, is not as serious as the interference by the degraded products. The actual loss of a significant portion of triacetyl compound by further reaction with acetic anhydride is over-compensated by the fact that the higher molecular weight peracetyl product is quite readily extracted by the official procedure. The formation of the peracetylation product, however, casts some doubt as to the validity of the polarimetric determination made directly on the extraction residue.

To test the relative merits of several analytical procedures available for determination of epinephrine in aqueous solution, analytical determinations were made by four independent methods on four

samples containing degraded epinephrine with apparent zero epinephrine content along with fairly pure drug. The methods studied included (*a*) direct ultraviolet absorption determination on the original solution, (*b*) modified U. S. P. XIV colorimetric method based on the use of ferro-citrate solution, (*c*) U. S. P. XV gravimetric method, and (*d*) the modified U. S. P. XV method based on acetylation time of three minutes with mechanical stirring and chromatographic isolation of triacetylepinephine from the reaction mixture. The results are shown in Table III.

Limitation of direct ultraviolet spectrophotometry to analysis of such degraded solutions is evident in Fig. 3. The upper spectral curves were obtained by direct ultraviolet spectrophotometry of degraded epinephrine solutions. The lower spectral curves represent corresponding triacetylepinephrine derivatives of the solutions prepared by the modified U. S. P. XV assay employing chromatographic isolation of the derivative. Values reported from the colorimetric procedure in Table III were the best obtainable after pretreatment of solutions with activated charcoal and filtration to remove color of degraded epinephrine. Results reported for both the ultraviolet and colorimetric method should properly apply only to the catechol chromophore or presence of phenolic hydroxyls. The methods are no more discriminating than this and tell nothing about the rest of the molecule or proportions of isomers. The official U. S. P. method gave high results in every instance as expected. The modified procedure, on the other hand, appears to give valid answers in every case.

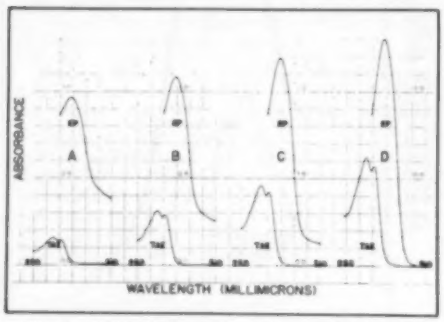



Fig. 3.—Ultraviolet spectra of several epinephrine solutions containing varying amounts of degraded products. The curves marked EP were obtained by measurements directly on fixed dilutions of these solutions. The lower curves marked TAE refer to measurements on TAE fraction isolated by chromatography. The compositions of the solutions were

| Sample No. | Epinephrine, % | Degraded Epinephrine, % |
|---|---|---|
| A | 0.050 | 0.150 |
| B | 0.100 | 0.100 |
| C | 0.150 | 0.050 |
| D | 0.200 | 0.000 |

Polarimetric determinations on the acetylated extract following the official procedure gave, as expected, erroneous but interesting results. This is evident in Table IV. Because the recovered weight of the acetylation product was always much higher than theoretical for all samples containing degraded epinephrine the official method of calculation is, of course, meaningless. It is interesting to note, however, if all of the optical activity is attributed to *l*-triacetylepinephrine and that no other optically active compound is present, a rather nice agreement with theory is obtained. Since in the official procedure TAE is totally contained in the extract, such a result may be expected if the net optical effect of peracetylation on the system was small. This is true because in these particular samples no racemization existed. For regular samples, however, such an assumption cannot safely be made and the modified procedure presented in this report must precede any polarimetric determination.

## RECOMMENDATION

It is recommended that the modified procedure described in this report be adopted as the official

TABLE III.—COMPARISON OF ASSAY METHODS IN ANALYSIS OF DEGRADED EPINEPHRINE SOLUTIONS

| Solutions | Composition, mg./100 ml.: Degraded Material | Composition, mg./100 ml.: Synthetic *l*-Epinephrine[a] | Epinephrine Found, mg./100 ml.: U. V. | Epinephrine Found, mg./100 ml.: Colorimetric | Epinephrine Found, mg./100 ml.: U. S. P. XV,[b] Gravimetric | Epinephrine Found, mg./100 ml.: Modified Procedure[c] |
|---|---|---|---|---|---|---|
| A | 150 | 50 | 146 | 62 | 81.4 | 49.3 |
| B | 100 | 100 | 166 | 106 | 129.0 | 100.5 |
| C | 50 | 150 | 180 | 156 | 162.5 | 148.5 |
| D | ... | 200 | 199 | 202 | 199.0 | 197.5 |

[a] Commercial high purity epinephrine with estimated purity above 98%.
[b] Values based on gravimetric determination of residue: ep. = wt. of residue × 0.5923.
[c] Values reported as *levo*-epinephrine.

TABLE IV.—GRAVIMETRIC AND POLARIMETRIC VALUES OBTAINED IN U. S. P. XV ASSAY

| Soln.[a] | Wt. of Acetylated Product, Gm.: Calcd. | Wt. of Acetylated Product, Gm.: Found | Recovery, %: Gravimetric | $\alpha^b$ | $(\alpha)^{25°}_{D}$ | *l*-Epinephrine, mg. in 30 ml. Dilution: Present | *l*-Epinephrine, mg. in 30 ml. Dilution: Found[c] | *l*-Epinephrine, mg. in 30 ml. Dilution: Found[d] |
|---|---|---|---|---|---|---|---|---|
| A | 0.0127 | 0.0206 | 162 | −0.23 | −56.0 | 7.5 | 9.8 | 7.3 |
| B | 0.0253 | 0.0327 | 129 | −0.46 | −70.5 | 15.0 | 17.1 | 14.5 |
| C | 0.0380 | 0.0412 | 108 | −0.72 | −87.8 | 22.5 | 23.8 | 22.7 |
| D | 0.0507 | 0.0504 | 99.5 | −0.95 | −94.3 | 30.0 | 30.1 | 29.8 |

[a] 15 ml. of solution diluted to 30 ml. with pH 4, 0.1 molar acetate buffer for analysis. (See Table III for composition.)
[b] Determined in 1-dm. micro tube.
[c] Calculated with expression: Wt. ep./30 ml. = $[0.5 + 0.5(\alpha)^{25}_{D}/-93] \times [(\text{Wt. Residue})(0.5923)]$.
[d] Calculated with expression: Wt. ep./30 ml. = $30 \times (\alpha^b - 0.95)$.

method for determination of epinephrine. The procedure appears to give valid results in all instances where it has been applied. It is not based on compensatory errors arising in the system. The method is theoretically sound in that results are directly related to quantitative conversion and isolation of the drug component into a definitely established derivative. Substitution of a chromatographic step in lieu of the exhaustive extraction in the official procedure, moreover, leads to an appreciable saving in operational time.

## REFERENCES

(1) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 260.
(2) Welsh, L. H., THIS JOURNAL, **44**, 507(1955).
(3) Higuchi, T., and Patel, K. P., *ibid.*, **41**, 171(1952).
(4) "United States Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 216.

# The Solubility and Complexing Properties of Oxytetracycline and Tetracycline III*

## Interactions in Aqueous Solution With Model Compounds, Biochemicals, Metals, Chelates, and Hexametaphosphate

By TAKERU HIGUCHI and SANFORD BOLTON†

**The present study extends the investigation of complexing tendencies of oxytetracycline to (*a*) a series of isomer, multifunctional aromatic compounds, (*b*) certain biochemicals, (*c*) calcium and magnesium ions, both free and as chelates, and (*d*) hexametaphosphate. The relative importance of grouping and their location on the binding tendency has been demonstrated. Measurements carried out on purines, DNA, B vitamins, etc., show that such biochemicals are, in general, relatively strong complexing agents. Data on alkali earths suggest that complexed drugs may still undergo chelation. Hexametaphosphate appears to undergo very slight, if any, complexing reaction with either tetracycline or oxytetracycline at pH values between 5 and 8.**

THE ABILITY of the tetracycline antibiotics to form complexes with organic substances as well as metal ions has been established in previous studies (1–4). In the present communication the binding properties of tetracycline have been subjected to further examination in an effort to obtain a broader picture of the conditions necessary for complex formation. More specifically, model complexing agents were chosen to shed additional light on factors which may produce the highest degree of binding. The interaction of oxytetracycline with some compounds of biological interest was also investigated on the basis that these substances contain appropriate polar centers and that these systems might possibly represent a mechanism of action of oxytetracycline *in vivo*. In addition, the binding behavior of metal chelates for organic molecules and interactions of oxytetracycline and tetracycline with sodium hexametaphosphate were examined.

These studies were not particularly undertaken to find new complex species of tetracyclines but rather to provide further insight into the general phenomenon of molecular binding. The antibiotic was chosen for the purpose of this investigation because (*a*) it possesses a number of functional groups conducive to complex formation as indicated by earlier studies and (*b*) because of its great medicinal importance. The binding and associative properties of the drug are, also, of fundamental interest in that its microbiological and pharmacodynamic actions may be strongly affected by such behaviors.

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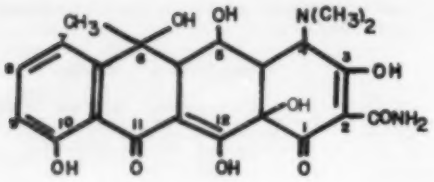
## STRUCTURAL CONSIDERATIONS

Because of the intricacy of these reactions and the complexity of the oxytetracycline molecule, it seems advisable, initially, to contemplate some factors which may affect these interactions. The extent of complex formation may be considered to be dependent, in part, on a series of competitive reactions. Accordingly, the intramolecular and intermolecular forces present in oxytetracycline would present a definite barrier to the formation of complexes. The low water solubility as well as the comparatively low melting point of oxytetracycline are probably manifestations of intramolecular hydrogen bonding. The hydroxy and carbonyl groups attached to carbons 10 and 11 and 12 and 1 are ideally situated for this type of bonding (Fig. 1). The fact that oxytetracycline forms a stable hydrate suggests a strong water-oxytetracycline bond which would interfere with an interaction of another species at the bonded sites.

Similarly, the complexing agent itself may be in-




OXYTETRACYCLINE

Fig. 1.—Oxytetracycline.

volved in a series of competitive reactions. An attraction for itself or water would hinder its tendency to complex with oxytetracycline. The fact that the complexing tendencies of the tetracyclines are greater in certain nonaqueous solvents (2), where at least competitive solvent interactions are lessened, seems to substantiate the above viewpoints. These competing reactions cannot be separated and measured in such a complex system, but the physical properties of the substances involved can present an insight into the relative magnitudes of these attractive forces.

## EXPERIMENTAL

**Reagents.**—Oxytetracycline dihydrate and tetracycline trihydrate were prepared by methods suggested by Gans and Higuchi (1). The purity was checked by solubility analysis. All other compounds were generally redistilled, recrystallized, or were of the highest purity commercially available. Sodium hexametaphosphate was used directly as supplied by Charles Pfizer and Co. Laboratories.

**Procedure.**—The experimental procedure was essentially the same as that used by Gans and Higuchi (1). An excess of antibiotic was equilibrated with 10 cc. of a solution of known concentration of complexing agent by shaking for eighteen hours at 25°. The solutions were maintained at pH 5 by use of a suitable buffer, and 0.1% sodium bisulfite was added as an antioxidant. In some cases, where the complexing agent was especially labile to oxidation, i. e., aminophenols, dihydroxy benzenes, and ascorbic acid, 1% sodium bisulfite was added and equilibration was carried out in an atmosphere of nitrogen. Clear aliquots were then appropriately diluted with pH 5 buffer for spectrophotometric analysis.

It should be noted in this respect that the condition of constant pH imposes certain restrictions on the experiment. At pH 5, oxytetracycline exists as a zwitterion and, therefore, the interaction form of oxytetracycline is necessarily limited to this species. Similarly, the acid-base ratio of any acidic or basic complexing agent will be fixed by the pH. In the absence of other factors, a homologous series of acids, for example, should show different degrees of activity depending on their ionization constants if either the acid or base species is active. Also, the buffer concentration has a definite effect on the solubility of oxytetracycline as shown in Fig. 2. This solubilization phenomenon was kept to a minimum by using a conveniently low buffer concentration.

**Analytical Methods.**—The amounts of oxytetracycline and tetracycline in solution were measured by convenient dilution in pH 5 buffer and subsequent spectrophotometric analysis utilizing the peaks at 353 mμ and 355 mμ, respectively. In certain cases, interference in this region of the spectrum by the complexing agent was unavoidable. Appropriate blank corrections were made at various wavelengths and checked by measuring the absorption of the solutions at these wavelengths. It was found that Beer's law relationship for oxytetracycline held at wavelengths from 350–380 mμ. In the analysis of oxytetracycline-riboflavin interaction, the minimum in the U. V. spectrum of riboflavin at 303 mμ was used to check the results obtained at 353 mμ.

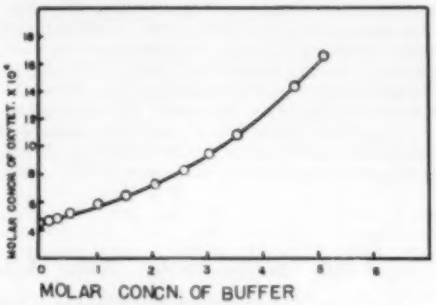



Fig. 2.—Phase diagram showing the effect of acetate buffer on the apparent solubility of oxytetracycline at 25°, pH 5.

Salicylaldehyde was found to interfere with the analysis in a manner which could not be blanked out. The solution after equilibration, in this instance, was made acid with hydrochloric acid and then extracted with three equal portions of ether. Most of the salicylaldehyde was removed in this manner. The small amount of oxytetracycline extracted was calculated and a correction made in the final analysis. This procedure was found to be accurate with solutions of known concentration of oxytetracycline and salicylaldehyde.

## RESULTS AND OBSERVATIONS

The general type interaction exhibited by lower concentrations of various compounds with oxytetracycline in aqueous solutions is shown in Fig. 3. Soluble complexes appeared to have formed in all cases studied. In the presence of excess crystalline drug, the apparent amount of the antibiotic in solution appears to increase often linearly with the concentration of the complexing agent. For such a system it is convenient to assume formation of a one-to-one complex and to calculate an equilibrium constant.

$$K_1 = \frac{(TX)}{(T)(X)} \qquad \text{(Eq. 1)}$$

where $(X)$ is the concentration of free complexing agent; $(T)$ is the concentration of free oxytetracycline; and $(TX)$ is the concentration of complex. The details of this calculation may be found in a paper by Higuchi and Zuck (5).

Figure 4 illustrates the effect of higher concentrations of many complexing agents. It is obvious that the order with respect to complexing agent is increasing at higher levels. Equilibrium constants

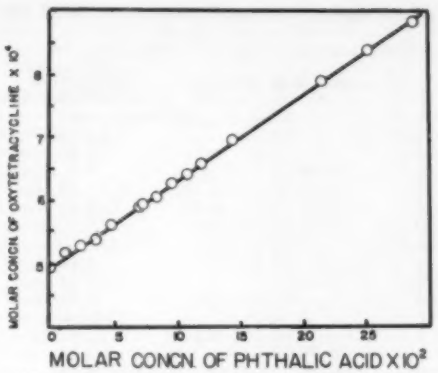



Fig. 3.—Phase diagram showing the effect of phthalic acid on the apparent solubility of oxytetracycline at 25°, pH 5.

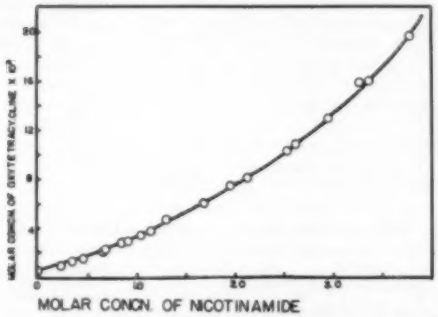



Fig. 4.—Phase diagram showing the effect of nicotinamide on the apparent solubility of oxytetracycline at 25°, pH 5.

were calculated for higher order interactions in the following manner:

$$K_2 = \frac{(TX_2)}{(X)(TX)} \qquad \text{(Eq. 2)}$$

$$K_3 = \frac{(TX_3)}{(X)(TX_2)} \qquad \text{(Eq. 3)}$$

Let

$$K_0 = \frac{\text{(Total Complex)}}{(T)(X)}$$

$$= \frac{(TX) + (TX_2) + (TX_3)}{(T)(X)}$$

$$= \frac{(TX)}{(T)(X)} + \frac{(TX_2)}{(T)(X)} + \frac{(TX_3)}{(T)(X)}$$

$$= K_1 + K_1K_2(X) + K_1K_2K_3(X)^2$$

Rearranging,

$$\frac{K_0 - K_1}{K_1(X)} = K_2 + K_2K_3(X) \qquad \text{(Eq. 4)}$$

A plot of $K_0 - K_1/K_1(X)$ vs. $(X)$ results in a straight line with slope $K_2K_3$ and intercept $K_2$. Typical results are shown in Fig. 5. In the cases of

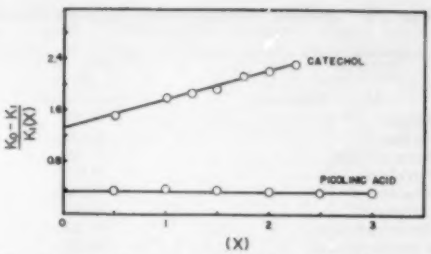



Fig. 5.—Plot illustrating the calculation of second and third order equilibrium constants.

*m*-hydroxybenzoic acid, salicylic acid, and pyrogallol, the solubility curves were described by $K_1$ and $K_2$ only, where $K_2$ equals $(TX_2)/(TX)(X)^2$. A modification of the above procedure was used to calculate $K_3$.

Although it cannot be shown that these equilibria are actually occurring in solution, these constants can accurately describe the solubility curves. To calculate the total concentration of oxytetracycline in solution at a particular concentration of complexing agent, the following method suffices:

Total oxytetracycline in solution =

$$(T) + (TX) + (TX_2) + (TX_3) \qquad \text{(Eq. 5)}$$

Combining Eqs. 1, 2, and 3 with Eq. 4

Total oxytetracycline in solution =

$$(T) + K_1(T)(X) + (T)K_1K_2(X)^2 + (T)K_1K_2K_3(X)^3 \qquad \text{(Eq. 6)}$$

Since $(X)$ is essentially equal to the total amount of complexing agent added to the system and $(T)$ is equal to approximately $5 \times 10^{-4}$ *M*, Eq. 6 can be reduced to the following form:

$$\text{Total oxytetracycline in solution} = 5 \times 10^{-4} \times [1 + K_1(X) + K_1K_2(X) + K_1K_2K_3(X)^2] \qquad \text{(Eq. 7)}^1$$

The slopes mentioned in Tables I, II, and III are merely the slopes of the straight lines obtained initially in the solubility measurements and are mentioned for convenience of comparison. The column headed "Complexing Agent Added" indicates the maximum amount of complexing agent added to the systems in these studies.

**Interactions of Oxytetracycline With Homologous Polar Aromatic Compounds.**—The purpose of this study was to note the effect of acidity and the presence of various polar groups, as well as their positions, on the complexing tendency with oxytetracycline. Table I shows that of the monofunctional compounds tested the carboxy group seemed to be more effective than either the aromatic nitrogen in pyridine or the amino group in aniline. In the series of dihydroxybenzene derivatives, resorcinol showed the greatest activity. The aminophenols and pyridine carboxylic acids showed a similar pattern: the *meta* isomer was stronger than the *para*, which in turn had a higher slope than the *ortho* isomer. Salicylic acid complexed most strongly in the series

[1] In the cases of pyrogallol, salicylic acid, and *m*-hydroxybenzoic acid where only $K_1$ and $K_2$ were necessary to describe the interaction, Eq. 7 reduces to:
Total Oxytet. = $5 \times 10^{-4}$ [1 + $K_1(X) + K_1K_2(X)^2$]
where $K_2 = (TX_2)/(X)^2(TX)$

TABLE I.—INTERACTION BETWEEN OXYTETRACYCLINE AND VARIOUS AROMATIC COMPOUNDS AT pH 5 AND 25°

| Compound | Slope × $10^2$ | Liters/Mole $K_1$ | Liters/Mole $K_2$ | Liters/Mole $K_3$ | Molar Concn. Complexing Agent Added |
|---|---|---|---|---|---|
| Phenol | 0.12 | 2.4 | ... | ... | 0.11 |
| Aniline | 0.06 | 1.2 | ... | ... | 0.105 |
| Benzoic acid | 0.16 | 3.2 | ... | ... | 0.21 |
| Pyridine | 0.06 | 1.2 | 0.85 | ... | 1.0 |
| Resorcinol | 0.225 | 4.5 | 1.00 | 0.45 | 4.05 |
| Catechol | 0.16 | 3.2 | 1.32 | 0.34 | 2.6 |
| Hydroquinone | 0.20 | 4.0 | ... | ... | 0.095 |
| *p*-Aminophenol | 0.10 | 2.0 | ... | ... | 0.12 |
| *o*-Aminophenol | 0.07 | 1.4 | ... | ... | 0.05 |
| *m*-Aminophenol | 0.13 | 2.6 | ... | ... | 0.11 |
| Salicylic acid | 0.32 | 6.4 | ... | 0.7 | 3.00[a] |
| *p*-Hydroxybenzoic acid | 0.28 | 5.6 | ... | ... | 0.075 |
| *m*-Hydroxybenzoic acid | 0.28 | 5.6 | ... | 2.3 | 0.41 |
| 2,4-Dihydroxybenzoic acid | 0.56 | 11.2 | 0.45 | ... | 0.48 |
| 2,5-Dihydroxybenzoic acid | 0.50 | 10.0 | ... | ... | 0.48 |
| 2,5-Dihydroxybenzoic ethanolamide | 0.54 | 10.8 | ... | ... | 0.38 |
| 3,4-Dihydroxybenzoic acid | 0.41 | 8.2 | 0.70 | ... | 0.52 |
| 3,5-Dihydroxybenzoic acid | 0.44 | 8.8 | ... | ... | 0.128 |
| *p*-Aminobenzoic acid | 0.18 | 3.6 | ... | ... | 0.084 |
| *o*-Aminobenzoic acid | 0.22 | 4.4 | ... | ... | 0.064 |
| *m*-Aminobenzoic acid | 0.17 | 3.4 | ... | ... | 0.03 |
| Nicotinic acid | 0.195 | 3.9 | ... | ... | 0.16 |
| Nicotinamide | 0.205 | 4.1 | 0.34 | ... | 3.9 |
| Isonicotinic acid | 0.155 | 3.1 | ... | ... | 0.10 |
| Picolinic acid | 0.13 | 2.6 | 0.31 | ... | 3.56 |
| Benzaldehyde | 0.14 | 2.8 | ... | ... | 0.030 |
| *m*-Hydroxybenzaldehyde | 0.26 | 5.2 | ... | ... | 0.078 |
| Salicylaldehyde | 0.14 | 2.8 | ... | ... | 0.037 |

[a] Calculated from data by Gans and Higuchi (1).

TABLE II.—INTERACTION OF VARIOUS SUBSTANCES WITH OXYTETRACYCLINE AT pH 5 AND 25°

| Compound | Slope × $10^2$ | Liters/Mole $K_1$ | Liters/Mole $K_2$ | Liters/Mole $K_3$ | Molar Concn. Complexing Agent Added |
|---|---|---|---|---|---|
| Ethyltheophylline | 0.74 | 14.8 | ... | ... | 0.127 |
| Gallic acid | 0.46 | 9.2 | ... | ... | 0.115 |
| 2,3-Diketo-1,2,3,4-tetrahydroxy-quinoxaline | 0.44 | 8.8 | ... | ... | 0.0017 |
| Apresoline HCl | 0.40 | 8.0 | ... | ... | 0.079 |
| *p*-Aminosalicylic acid | 0.39 | 7.8 | ... | ... | 0.12 |
| Melamine | 0.32 | 6.4 | ... | ... | 0.064 |
| Quinoline-8-carbox. acid | 0.27 | 5.4 | ... | ... | 0.024 |
| PEG 4000 | 0.26 | 5.2 | ... | ... | 0.06 |
| Pyrogallol | 0.22 | 4.4 | ... | 0.84 | 3.17 |
| Maleic hydrazide | 0.19 | 3.8 | ... | ... | 0.055 |
| 8-Hydroxyquinoline | 0.18 | 3.6 | ... | ... | 0.017 |
| Antipyrine | 0.17 | 3.4 | 0.36 | ... | 3.25 |
| *o*-Phthalic acid | 0.14 | 2.8 | ... | ... | 0.36 |
| Monomethyloldimethylhydantoin | 0.095 | 1.9 | 0.37 | ... | 2.8 |
| Pyridazine | 0.09 | 1.8 | ... | ... | 0.26 |
| Dimethylhydantoin | 0.07 | 1.4 | 0.43 | ... | 1.05 |
| Oxazolidone | 0.06 | 1.2 | 0.19 | 0.23 | 3.0 |
| Barbital | 0.06 | 1.2 | ... | ... | 0.042 |
| PEG 1500W | 0.06 | 1.2 | ... | ... | 0.18 |
| 3-Hydroxy-3-methyl-2-butanone | 0.012 | 0.24 | 1.2 | ... | 0.405 |
| Malonic acid | 0.01 | 0.2 | ... | ... | 0.96 |

of hydroxybenzoic acids and, similarly, the hydroxy derivatives of salicylic acid proved stronger than other dihydroxybenzoic acids tested. Again, in the aminobenzoic acid series, the *ortho* isomer showed greatest activity. It is only in the case of the hydroxybenzaldehydes that the *ortho* isomer seemed to be less active. In an attempt to correlate these results with complexing activity, relative acidity and the positions of relevant groups were considered.

A direct correlation between acidity (as measured by ionization constants) and activity is not apparent. This is most easily seen in the series of dihydroxybenzoic acids. At the pH of these experiments the dihydroxybenzenes are essentially all in the nondissociated form. It should also be noted in this respect that the slopes obtained for gentisic acid and nicotinic acid were slightly smaller than the slopes of their amide derivatives.

In each series of homologous compounds the influence on binding tendency of position isomerism is evident. In all cases, except the benzaldehyde series, the compounds with polar groups separated

TABLE III.—INTERACTION OF VARIOUS BIOCHEMICALS WITH OXYTETRACYCLINE AT pH 5 AND 25°

| Compound | Slope × 10² | Liters/Mole $K_1$ | Liters/Mole $K_2$ | Liters/Mole $K_3$ | Molar Concn. Complexing Agent Added |
|---|---|---|---|---|---|
| Riboflavin-5-phos. Na | 1.28 | 25.6 | ... | .. | 0.0014 |
| Desoxyribonucleic acid | 1.15 | 23.0 | ... | .. | 0.08 |
| Adenylic acid | 0.50 | 10.0 | ... | .. | 0.062 |
| Thymine | 0.31 | 6.2 | ... | .. | 0.037 |
| Uracil | 0.25 | 5.0 | ... | .. | 0.019 |
| Pyridoxine HCl | 0.23 | 4.6 | 0.26 | 0.6 | 1.03 |
| Dimethyluracil | 0.20 | 4.0 | 0.5 | .. | 1.16 |
| Alloxan | 0.25 | 5.0 | ... | .. | 0.14 |
| Thiamine HCl | 0.225 | 4.5 | 0.6 | 0.8 | 1.02 |
| Tryptophane | 0.20 | 4.0 | ... | .. | 0.01 |
| Cytosine | 0.15 | 3.0 | ... | .. | 0.059 |
| Creatinine | 0.13 | 2.6 | 0.35 | .. | 1.06 |
| *l*-Histidine HCl | 0.07 | 1.4 | 0.45 | .. | 0.89 |
| Ascorbic acid | 0.06 | 1.2 | ... | .. | 0.057 |
| Urea | 0.02 | 0.4 | 0.52 | .. | 7.3 |

by three carbon atoms seemed to be most active. Thus, if a more stable complex can be formed when two polar groups are ideally oriented so as to fit receptor sites in the oxytetracycline molecule, an optimum positional effect would be expected. The apparent anomaly in the case of salicylaldehyde can be rationalized on the basis of the very strong intramolecular hydrogen bonding existing in this compound (6).

It was noted, also, that the complexing properties of these multifunctional compounds are approximately additive. Accordingly, the slope of a hydroxybenzoic acid was close to the sum of the slopes of benzoic acid and phenol. Figure 6 shows a plot of expected slope value, calculated strictly on an additive basis, *vs.* the observed value. It is seen that although the properties are not strictly additive, there is a linear relationship in the series of compounds where polar groups are separated by 3 and 4 carbons. The low deviation from this additive effect in the aminophenols could be due to strong intermolecular associations in solution. The high deviations in the cases of the dihydroxybenzoic acids could be explained on the basis of a greater possibility of multiple point contacts, which would produce a more stable complex.

**Interactions of Oxytetracycline With Other Simple Polar Molecules of Various Configurations.**—Table II lists some additional compounds which were used in this study. They were chosen not only to help elucidate the data on the acids but also to note the effect of different types of configurations on complexing activity.

8-Hydroxyquinoline, 8-carboxyquinoline, the quinoxaline derivative, and apresoline hydrochloride all showed greater activity than was expected. Although the reason for this would be difficult to interpret, it should be noted that these substances all contain fused ring systems. Andrews has mentioned that the stability of 1–1 complexes increases with the number of rings in the complexing agent (7).

Both *p*-aminosalicylic acid and gallic acid behaved approximately as expected. The lower slope of gallic acid and pyrogallol seems to substantiate the importance of position of polar groups. A similar observation was made by Sakai in an investigation of the effect of various benzene derivatives on the solubility of riboflavin (8). Phthalic acid exhibited relatively weak binding properties.

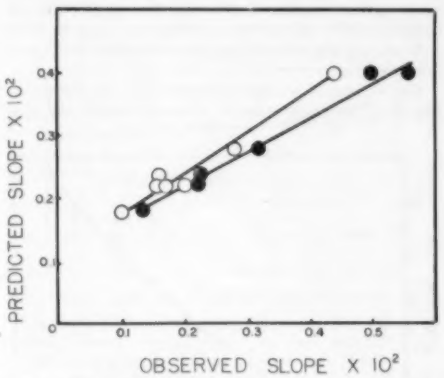



Fig. 6.—Predicted slope value *vs.* observed slope value where polar centers are separated by three carbons (●) and four carbons (O).

In contrast to aromatic compounds, purely aliphatic compounds show relatively low affinity for the antibiotic. Both malonic acid, which contains the optimum "three carbon separation," and hydroxymethylbutanone had a very small effect on the solubility of oxytetracycline. The behaviors of barbital, oxazolidone, and the hydantoins are also similarly weak. This could be explained on the basis of Pi complexing or the necessity of a planar structure to obtain proper orientation.

**Interactions of Oxytetracycline With Compounds of Biological Interest.**—Table III lists some metabolites whose configurations seemed to be appropriate for complex formation with oxytetracycline. Oxytetracycline was found, for example, to have an effect on the excretion of B vitamins (9) and their activity (10). The investigation of these systems was also indicated on the basis that the biological action of oxytetracycline may conceivably involve association with essential metabolites *in vivo*.

Fused ring compounds such as desoxyribonucleic acid, adenylic acid, and riboflavin exhibited the strongest binding tendencies of the compounds in this series. Tryptophane, which also falls into this class, showed similar high order of complexing ability. Although other interesting biochemical compounds such as uric acid, folic acid, rutin, and quercitin were all considered, they were found to be

so insoluble as to make any accurate observations impossible.

The results in the uracil series are interesting as they show the effect of substituted nonpolar groups on complexing activity. Uracil appears to be less active than thymine, and dimethyluracil less active, in turn, than uracil. The fact that these biochemicals do associate with oxytetracycline is not meant, of course, to infer that these interactions *per se* are important in the physiological activity of the antibiotic. Rather, the idea that these interactions do occur with such naturally occurring substances is of interest in that similar interactions may take part in the actions of such drugs.

**Investigation of the Interaction of Metal Chelates of Oxytetracycline With Some Organic Molecules.**—Figure 7 illustrates the effect of $CaCl_2$ and $MgCl_2$ on the solubility of oxytetracycline. Although the existence and properties of these chelates have been investigated elsewhere (11, 3), the possibility of metal chelates of oxytetracycline reacting in turn with an organic complexing agent seemed worthy of investigation.

The results of a typical experiment are shown in Fig. 8. The concentration of metal ion in the system was kept constant while the concentration of monomethyloldimethylhydantoin (MDH) was varied. The increase in solubility of oxytetracycline was noted beyond that calculated on an additive basis, i. e., independent interaction of metal and MDH added. A similar plot, keeping MDH constant and varying the concentration of metal ion, results, also, in a straight line. It is obvious from these plots that the increase in solubilization of oxytetracycline is proportional to the concentration of both metal and organic species. The relationship can be expressed by Eq. 8.

$$\text{Increase in solubility beyond expected} = K\,(\text{concentration of metal ion})\;X\;(\text{concentration of comp. agent})\;(\text{Eq. 8})$$

where $K$ is an appropriate constant.

This relationship was tested at higher concentrations of metal and complexing agent, and the results as well as the "$K$" calculated from Eq. 8 are listed in Table IV. (In the oxazolidone-magnesium chloride system, a plot similar to Fig. 8 produced a curve which deviated from linearity at higher concentrations. An extrapolation of this curve was used to calculate the expected solubility of oxytetracycline at still higher concentrations.) This solubilization phenomenon is probably due to a new association species resulting from the interaction of the oxytetracycline chelate or the oxytetracycline-organic complexing agent association product with the free metal or organic compound.

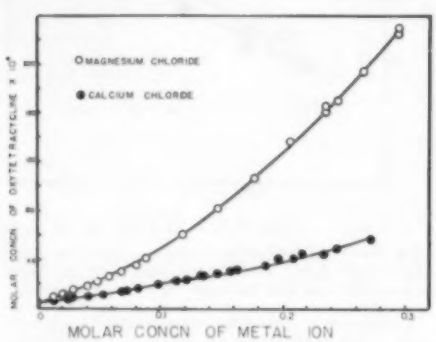



Fig. 7.—Phase diagram showing the effect of magnesium and calcium chlorides on the apparent solubility of oxytetracycline at 25°, pH 5.

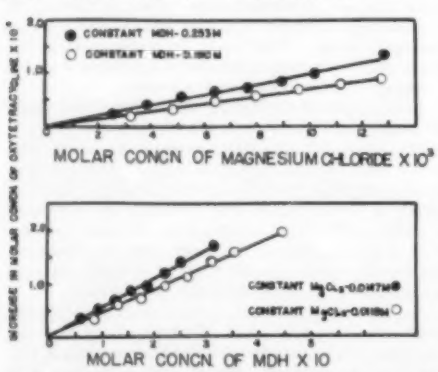



Fig. 8.—Plot showing the observed increase in the solubility of oxytetracycline beyond that calculated on an additive basis in the system magnesium chloride-monomethyloldimethylhydantoin-oxytetracycline at 25°, pH 5.

These promising results suggested further investigation of similar systems involving a chelating agent, metal ion, and oxytetracycline. One of two results was expected: (*a*) the solubility of oxytetracycline would be reduced because of competition for the metal ion by the chelating agent, or (*b*) a new reaction would occur and the solubility of oxytetracycline would be significantly increased.

In the presence of ascorbic acid and magnesium chloride the solubility of oxytetracycline was increased only a small amount above that calculated on an additive basis. In view of the above discussion, this result was unexpected. However, a potentiometric titration of ascorbic acid in the presence of magnesium chloride compared to a similar titration in which magnesium chloride was replaced by sodium chloride of equal ionic strength demonstrated that no chelate formation occurs in this system, at least in the pH range 3–8. Thus, the small increase in solubility of oxytetracycline observed seems to be due only to the type of reaction exhibited in the three-component systems previously discussed.

It can be seen from Fig. 9 that the solubility of oxytetracycline is significantly reduced when EDTA is added to the system oxytetracycline-magnesium chloride. EDTA, alone, had a negligible effect on the solubility of oxytetracycline. Using the data of Schwarzenbach (12, 13), the amount of metal ion in solution in the presence of EDTA at pH 5 was calculated. The solubility of oxytetracycline observed did not correspond to the solubility expected from the above calculation but the presence of buffer as well as the competing oxytetracycline-metal interaction probably invalidates the use of Schwarzenbach's data here. It is to be noted that the decrease in solubility of oxytetracycline with rising pH

TABLE IV.—INTERACTIONS OF CALCIUM AND MAGNESIUM IONS WITH ORGANIC COMPLEXING AGENT AND OXYTETRACYCLINE AT pH 5 AND 25°

| Molar Concn. Organic Compound | | Molar Concn. Metal Ion | Liters/Mole K | Calculated Increase in Molar Concn. Oxytet. × 10⁴ | Observed Increase in Molar Concn. Oxytet. × 10⁴ |
|---|---|---|---|---|---|
| Monomethyloldimethylhydantoin | 0.675 | Mg 0.038 | 0.038 | 21.3 | 21.2 |
| | 1.038 | 0.098 | | 38.6 | 41.7 |
| | 1.210 | 0.118 | | 54.3 | 57.2 |
| | 0.51 | 0.0494 | | 9.6 | 7.5 |
| | 0.38 | 0.0365 | | 5.2 | 6.7 |
| Oxazolidone | 0.35 | 0.0498 | 0.39[a] | 7.8 | 8.3 |
| | 1.00 | 0.06 | | 28.1 | 24.0 |
| | 1.05 | 0.076 | | 40.5 | 39.7 |
| | 0.66 | 0.116 | | 44.2 | 39.2 |
| | 1.44 | 0.106 | | 80.7 | 84.3 |
| Resorcinol | 0.30 | 0.067 | 0.115 | 23.2 | 24.3 |
| | 0.448 | 0.158 | | 81.5 | 80.0 |
| | 0.605 | 0.091 | | 63.4 | 71.0 |
| Ethyltheophylline | 0.975 | Ca 0.0997 | 0.22 | 21.4 | 22.0 |
| | 1.00 | 0.148 | | 32 6 | 32.3 |

[a] At very low concentrations of $Mg^{++}$ and oxazolidone.

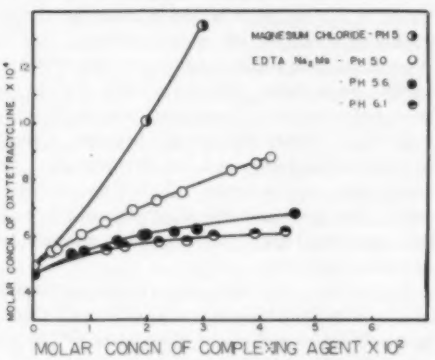

MAGNESIUM CHLORIDE - PH 5
EDTA Na2Mg - PH 5.0
PH 5.6
PH 6.1
MOLAR CONCN OF OXYTETRACYCLINE X 10⁴
MOLAR CONCN OF COMPLEXING AGENT X 10²

Fig. 9.—Phase diagram showing the effect of $Na_2Mg$ EDTA on the apparent solubility of oxytetracycline at 25° at several pH's. The effect of magnesium chloride at pH 5 is included for comparison.

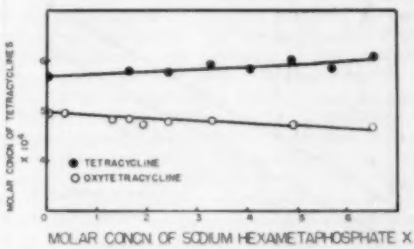



Fig. 10.—Phase diagram showing the effect of sodium hexametaphosphate on the solubility of tetracycline and oxytetracycline at 25°, pH 5.

is to be expected if the decrease in solubility is merely due to the removal of metal ion by EDTA.

**Interaction Studies of Sodium Hexametaphosphate With Tetracycline and Oxytetracycline.**—Recent commercial interest in tetracycline-hexametaphosphate combinations in various dosage forms prompted an investigation of a possible interaction in this system. A complex of sodium hexametaphosphate and tetracycline, isolated at pH 1.5 (14) and confirmation of the increased oral absorption of this combination (15, 16) have been reported in the recent literature.

The results of solubility studies at pH 5 as depicted in Fig. 10 strongly suggest that only minimal interaction occurs in these systems. Similar results were obtained at pH 7. To check the possibility that these negative results were not due to certain anomalies in the experimental setup, such as interference by the buffers used, a variation was introduced. The solutions of sodium hexametaphosphate and antibiotics were equilibrated in distilled water, the pH of each solution being checked. The results of this study with tetracycline, including variations in pH with concentration of sodium hexametaphosphate, are shown in Fig. 11. Similar results were obtained with oxytetracycline. The solubility and pH curves seem to parallel each other indicating that the change in solubility might be a function of pH only.

In conjunction with this study, the solubility of the tetracyclines was determined in the absence of buffer by extrapolating curves of antibiotic solubility *vs.* buffer concentration to zero buffer concentration at several pH's between seven and eight. The results are shown in Fig. 12. By subtracting the solubility as observed in Fig. 12 from that observed in Fig. 11 at that particular pH, the increase in solubility of tetracycline as a function of hexametaphosphate concentration was calculated. The results, thus obtained, in both oxytetracycline and tetracycline systems are depicted in Fig. 13. Although this plot seems to indicate that there is some interaction occurring, the errors inherent in the approximations introduced do not permit the use of these data as a means of analyzing the interaction.

The fact that comparatively little binding tendency was noted suggests that sodium hexametaphosphate must act in some other manner if the rate of absorption is actually increased in its presence. Thus, for example, increased oral absorption of this combination may be elicited through a membrane effect in addition to the sequestering behavior of sodium hexametaphosphate. The latter action probably accounts largely for the observed effect,

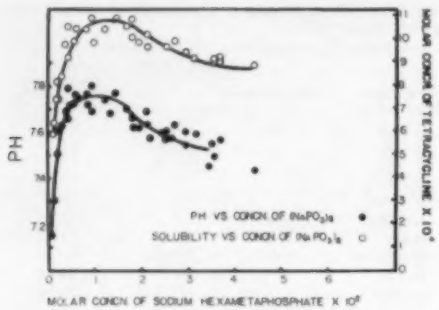

PH
MOLAR CONCN OF TETRACYCLINE X 10⁴
PH VS CONCN OF (NaPO₃)₆
SOLUBILITY VS CONCN OF (NaPO₃)₆
MOLAR CONCN OF SODIUM HEXAMETAPHOSPHATE X 10²

Fig. 11.—Diagram showing the effect of sodium hexametaphosphate on the solubility of oxytetracycline and the pH of the resulting solutions in the absence of buffer at 25°.

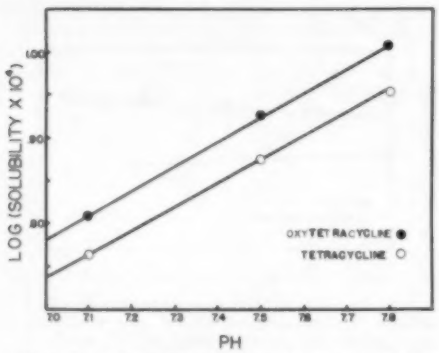



Fig. 12.—Plot showing the variation in solubility of tetracycline and oxytetracycline with pH at 25° in the absence of buffer.

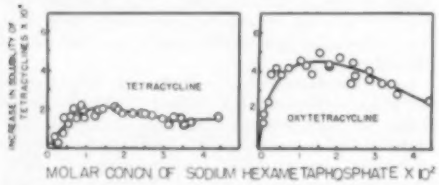

INCREASE IN SOLUBILITY OF TETRACYCLINES X 10⁴
TETRACYCLINE
OXYTETRACYCLINE
MOLAR CONCN OF SODIUM HEXAMETAPHOSPHATE X 10²

Fig. 13.—Plot showing the increase in solubility of the tetracyclines *vs.* concentration of sodium hexametaphosphate in the absence of buffer at 25°.

## SUMMARY

1. The results of the interaction of a series of homologous aromatic hydroxy, amino, carboxylic acid, and pyridine compounds with oxytetracycline seem to indicate that there exists a relationship between activity and position of functional groups. There also seems to be a correlation between activity and the number and type of polar groups present.

2. Aromaticity seems to be essential for optimum complexing activity as determined by experiments with a variety of nonaromatic compounds. Also the presence of fused ring systems seems to enhance complexing activity. There is a suggestion of Pi complexing playing an important role in these interactions.

3. Oxytetracycline forms complexes with a variety of metabolites, vitamins, and amino acids. These interactions suggest a possible mechanism of action *in vivo*.

4. A combination of calcium or magnesium ions and various organic complexing agents resulted in a synergistic action as noted by the increase in solubility of oxytetracycline. EDTA decreased the apparent solubility of the oxytetracycline-magnesium chelate probably due to removal of metal ion in a competitive reaction.

5. Only little interaction between sodium hexametaphosphate and oxytetracycline and tetracycline was observed. This is not meant to imply that increased oral absorption of this combination does not occur via other physical or chemical pathways.

6. At best, only suggestions of the mechanism of action of these interactions can be made due to the complex nature of the systems as well as the presence of inseparable competing reactions.

## REFERENCES

(1) Gans, E. H., and Higuchi, T., THIS JOURNAL, **46,** 458 (1957).
(2) Gans, E. H., and Higuchi, T., *ibid.*, **46,** 587(1957).
(3) Albert, A., and Rees, C. W., *Nature*, **177,** 433(1956).
(4) Albert, A., *ibid.*, **172,** 201(1953).
(5) Higuchi, T., and Zuck, D. A., THIS JOURNAL, **42,** 138 (1953).
(6) Pauling, L., "The Nature of the Chemical Bond," Cornell University Press, N. Y., 1940, p. 319.
(7) Andrews, L. J., *Chem. Rev.*, **54,** 713(1954).
(8) Sakai, K., *Nagoya J. Med. Sci.*, **18,** 237(1956).
(9) Di Raimondo, F., Angerano, D., and Mannino, N., *Chem. Abstr.*, **49,** 4103(1955).
(10) Dony-Crotteaux, J., *J. pharm. Belg.*, **12,** 179(1957).
(11) Regna, P. P., *et al.*, *J. Am. Chem. Soc.*, **73,** 4211(1951).
(12) Schwarzenbach, G., *Helv. Chim. Acta*, **30,** 1798(1947).
(13) Schwarzenbach, G., *ibid.*, **31,** 1029(1948).
(14) Kaplan, M. A., *et al.*, *Antibiotic Med. & Clin. Therapy*, **4,** 99(1957).
(15) Pulaski, E. J., and Isokane, R. K., *ibid.*, **4,** 408(1957).
(16) Cronk, G. A., Naumann, D. E., and Casson, K., *Antibiotics Ann.*, **1957–58,** 397.

# Isotonic Solutions IX*

## The Permeability of Red Corpuscles to Some Monohydric and Polyhydric Alcohols

By PAUL ZANOWIAK† and WILLIAM J. HUSA

**The degree of hemolysis of rabbit and human erythrocytes in solutions of a series of monohydric and polyhydric alcohols was investigated. When possible, these data were used to calculate van't Hoff *i* values. The monohydric and polyhydric alcohols which had a molecular weight less than that of erythritol caused complete hemolysis in concentrations calculated to be isosmotic. Certain concentrations of the monohydric alcohols caused hemolysis even in the presence of 0.6 per cent (0.1 *M*) sodium chloride. The low molecular weight diols and triol investigated did not cause hemolysis in the presence of 0.6 per cent sodium chloride. The polyhydric alcohols of higher molecular weight prevented hemolysis. It appeared that the phenomena occurring at the erythrocyte membrane in the case of the alcohols might be influenced by molecular weight and the number of hydroxyl groups present; the ratio of hydroxyl groups to the number of carbon atoms in the molecule might be important.**

IN AN INVESTIGATION of isotonic solutions of various compounds, Husa and Adams (1) showed that certain of these, including several alcohols, did not prevent hemolysis at concentrations which were calculated to be isotonic according to physicochemical data. Grosicki and Husa (2) stressed the importance of making hemolytic tests, especially as a check on physicochemical data, for the preparation of parenteral solutions. They devised a hemolytic method for determining sodium chloride equivalents and calculated isotonic coefficients for various amino acids, sugars, and salts. Husa and co-workers (3–6), employing the hemolytic method, studied the effects of urea and urea derivatives, numerous electrolytes, salts of organic acids, and alkaloidal salts upon rabbit and human red blood cells.

Many organic hydroxy compounds have been found to penetrate erythrocytes of various species. However, it has been shown that some sugars did not possess this ability (2). The purpose of this investigation was to determine, wherever possible, the isotonic coefficients of a series of alcohols and to obtain data that might contribute to a hypothesis which could correlate the molecular weight and chemical configuration of alcohols with their erythrocyte membrane activity. A hemolytic method was used with rabbit and human red blood cells.

### EXPERIMENTAL

**Collection of Blood.**—The blood samples employed in the present investigation were obtained and treated in the same manner as described previously by Husa and co-workers (2, 3). Human blood samples were obtained mainly from the veins of the arms of a twenty-four year old male. Several experiments were conducted with venous blood of other donors.

**Preparation of Solutions.**—Stock solutions of various concentrations of the alcohols were prepared by weighing the proper amounts of the respective alcohols and bringing to volume with triple-distilled water. Sodium chloride solutions were prepared in the same way. Necessary dilutions were then made from these stock solutions in the manner described previously (3), as were the solutions containing both sodium chloride and an alcohol.

**Quantitative Determination of Per Cent Hemolysis.**—The method used to determine the degree of hemolysis of erythrocytes in the various solutions involved in this investigation was essentially that of Hunter (7) and has been described in detail by Grosicki and Husa (2). The major difference in procedure in the present investigation was that all experiments were carried out at a temperature of 37° ± 0.1°.

**Calculation of *i* Values.**—When the concentrations of sodium chloride and any other compound needed to cause a certain degree of hemolysis are known, the isotonic coefficient (*i* value) of the other compound can be calculated according to the osmotic equation used by Grosicki and Husa (2). This was the basis for the *i* value calculations in the present investigation.

In experiments carried out in the presence of 0.2% sodium chloride, the concentration of sodium chloride used in the osmotic equation was obtained by subtracting 0.2 Gm./100 ml. sodium chloride from values determined by interpolation of curves (concentration *vs.* per cent hemolysis) drawn for each blood sample (6).

### DISCUSSION

**Variability of Blood.**—The importance of the variability factor of blood samples used in quantitative hemolytic determinations has been discussed by Husa and co-workers (5, 6). A range of concentration of sodium chloride solutions was employed in this investigation as a standard of reference to cir-

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cumvent error ascribable to the variability of erythrocyte fragility. The degree of hemolysis occurring in the various concentrations of sodium chloride was determined for each blood sample and differences in erythrocyte fragility were overcome by comparison of these values with those found for the degrees of hemolysis in solution of the substance under investigation.

Procedural features that were utilized to diminish experimental error were the use of duplicative tubes of all solutions within each experiment and the use of five separate tubes for 100% hemolysis for each blood sample; three of these tubes were placed at the beginning and two at the end of each run. For each compound studied, at least two experiments, utilizing two different blood samples of the same species, were performed.

**Value of *i* for Sodium Chloride.**—Previous hemolytic determinations (1–6) were carried out at 25° and the *i* values were calculated using sodium chloride as the standard and 1.86 as the *i* value for sodium chloride. The present investigation was carried out at 37°. It, therefore, became necessary to determine whether the *i* value for sodium chloride would be different at this temperature than at 25° as used by previous workers.

From electromotive and boiling point data, the osmotic coefficient of sodium chloride from 0 to 40° and from 0.1 *M* to 4.0 *M* has been computed and graphically summarized by Harned and Owen (8). For 0.1 *M* sodium chloride there was no significant change of the osmotic coefficient from 0 to 40°, when derived from such data.

It also seemed desirable to obtain hemolytic data which could validate the use of 1.86 as the *i* value for sodium chloride at 37°. Therefore, an experiment was designed employing the general range of sodium chloride concentrations used in conjunction with rabbit blood in this investigation. The procedure was essentially the same as described above except that four tubes of each concentration were used; two each were kept at 25° for forty-five minutes, while two were maintained at 37° for the same period. The tubes held at 25° were regarded as the "known" factor for this experiment and the value for *i* at that temperature was taken as 1.86. From the data so obtained, a value of *i* for sodium chloride at 37° was determined. The results indicated that the *i* values for sodium chloride obtained at 37° were directly comparable with the data obtained at 25° by previous workers and 1.86 was used as the *i* value for sodium chloride in this investigation.

**Results with Monohydric Alcohols.**—Quantitative hemolytic determinations were performed with methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *sec*-butyl and *tert*-butyl alcohols, employing rabbit and/or human blood. The general range of concentration of the solutions of the monohydric compounds was 0.5 to 10% except where the respective solubilities prevented this, in which case the highest practical concentrations were used, e. g., 4% (0.5*M*) of isobutyl alcohol. Other exceptions occurred when after mixing with blood, precipitates formed at the higher concentrations employed, as with *n*-propyl and *n*-butyl alcohols. In these cases the highest practical concentrations were also employed.

In each determination involving the monohydric compounds the highest concentration used was well above that needed to give the same freezing point as a 0.5% solution (0.1 *M*) of sodium chloride. Such a solution might be expected to prevent hemolysis of rabbit and human erythrocytes. However, the results indicated that approximately 100% hemolysis occurred with such concentrations, as well as with the lower concentrations investigated. These data seemed to agree with the results of Griyns (9) who noted that chicken and horse erythrocytes hemolyzed in the presence of aliphatic monohydric alcohols and glycerin. Booij, *et al.* (10), found that normal alcohols at higher concentrations produced hemolysis.

The results obtained from determinations involving certain concentrations of various monohydric alcohols in the presence of 0.2% (0.03 *M*) and 0.6% (0.1 *M*) sodium chloride are summarized in Tables I and II respectively. Table II indicates that all the monohydric alcohols investigated, except methyl alcohol, produced hemolysis in the presence of 0.6% sodium chloride. The effect of a range of concentration of each of these compounds upon human and rabbit erythrocytes in the presence of 0.6% sodium chloride was also investigated.

In the case of ethyl alcohol in the presence of 0.6% sodium chloride, solutions of 5% (1.1 *M*) or less did not cause hemolysis of rabbit and human blood. However, 15% (3.3 *M*) solutions produced complete hemolysis. For *n*-butyl alcohol in the presence of 0.6% sodium chloride, solutions of 1.5% (0.2 *M*) or less did not cause hemolysis of rabbit or human erythrocytes, but 2% solutions (0.3 *M*) produced complete hemolysis.

When rabbit erythrocytes were employed, solu-

TABLE I.—EFFECT OF VARIOUS MONOHYDRIC ALCOHOLS ON HUMAN AND/OR RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.2% SODIUM CHLORIDE

| | Rabbit Erythrocytes | | Human Erythrocytes | |
|---|---|---|---|---|
| Compound | % | Hemolysis, Approximate % | % | Hemolysis, Approximate % |
| Methyl alcohol | .. | .. | 10 | 100 |
| Ethyl alcohol | 10 | 100 | 10 | 100 |
| *n*-Propyl alcohol | 7 | 100 | 7 | 100 |
| Isopropyl alcohol | 10 | 100 | .. | .. |
| *n*-Butyl alcohol | 2 | 100 | 2 | 100 |
| Isobutyl alcohol | 4 | 100 | .. | .. |
| *sec*-Butyl alcohol | 5 | 100 | .. | .. |
| *tert*-Butyl alcohol | 10 | ppt. | .. | .. |

TABLE II.—EFFECT OF VARIOUS MONOHYDRIC ALCOHOLS ON HUMAN AND/OR RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE

| | Rabbit Erythrocytes | | Human Erythrocytes | |
|---|---|---|---|---|
| Compound | % | Hemolysis, Approximate % | % | Hemolysis, Approximate % |
| Methyl alcohol | .. | .. | 10 | 0 |
| Ethyl alcohol | 10 | 70 | 10 | 70 |
| *n*-Propyl alcohol | 7 | 100 | 7 | 100 |
| Isopropyl alcohol | 10 | 100 | .. | .. |
| *n*-Butyl alcohol | 2 | 100 | 2 | 100 |
| Isobutyl alcohol | 4 | 100 | .. | .. |
| *sec*-Butyl alcohol | 5 | 100 | .. | .. |
| *tert*-Butyl alcohol | 10 | ppt. | .. | .. |

tions of 2% (0.3 *M*) or less, of *n*-propyl alcohol in the presence of 0.6% sodium chloride did not cause hemolysis, but 5% solutions (0.8 *M*) produced complete hemolysis. With human erythrocytes, 4% solution (0.7 *M*) or less did not cause hemolysis, while 5% (0.8 *M*) solution produced complete hemolysis.

In the presence of 0.6% sodium chloride, solutions of 5% (0.8 *M*) of isopropyl alcohol did not cause hemolysis of rabbit erythrocytes, but solutions of 10% (1.7 *M*) produced complete hemolysis. Solutions of isobutyl alcohol of 1.5% (0.2 *M*) or less in the presence of 0.6% sodium chloride did not cause hemolysis; however, 2% solutions (0.3 *M*) caused approximately 60% hemolysis. Solutions of 2% (0.3 *M*) of *sec*-butyl alcohol in the presence of 0.6% sodium chloride caused approximately 5% hemolysis of rabbit blood, while 4 and 5% solutions (0.5 *M* and 0.7 *M*) caused complete hemolysis. Similarly, 5% solutions (0.7 *M*) of *tert*-butyl alcohol in the presence of 0.6% sodium chloride caused approximately 5% hemolysis, whereas 7% solutions (0.9 *M*) caused complete hemolysis.

In the case of the monohydric alcohols which were found to produce hemolysis in the presence of 0.6% sodium chloride, the degree of hemolysis seemed to increase with an increase in the concentration of the respective compounds. For each of these compounds, as outlined in the above discussion, there appeared to be a certain critical concentration below which, in the presence of 0.6% sodium chloride, hemolysis did not occur.

At certain higher concentrations of these alcohols, precipitates formed when 0.6% sodium chloride was present. This precipitation may have been due to denaturation of one or more of the proteins of the blood.

**Results with Certain Polyhydric Alcohols.**—Quantitative determinations were carried out employing ethylene glycol, 1,2-propanediol, glycerol, and 1,3-butanediol in concentrations of 0.5 to 10%. In each determination involving these compounds, the 10% solution was well above the concentration needed, according to osmotic calculations, to have the same osmotic pressure as a 0.5% (0.1 *M*) solution of sodium chloride. The results indicated that approximately 100% hemolysis occurred with this concentration, as well as with lower concentrations investigated.

Abnormal permeability of erythrocytes in solutions of some nonelectrolytes can be corrected in some cases by the addition of a small proportion of an electrolyte such as sodium chloride (2). The effect of the presence of 0.2% (0.03 *M*) and 0.6% (0.1 *M*) sodium chloride upon the hemolysis by ethylene glycol, 1,2-propanediol, glycerol, and 1,3-butanediol has been studied in the present investigation. In each case, 10% solutions of the respective compounds were used. Regardless of the type of erythrocytes employed, all of these solutions caused complete hemolysis in the presence of 0.2% sodium chloride. In the presence of 0.6% sodium chloride the 10% solutions [(1.1 *M*) to (1.6 *M*)] of each of these compounds did not cause hemolysis.

**Results with Other Polyhydric Alcohols.**—It was possible to determine *i* values for certain of the polyhydric alcohols investigated. These compounds and the average *i* values found for each, are given in Tables III and IV which also include their *i* values determined in the presence of 0.2% (0.03 *M*) sodium chloride. These data seem in agreement with several investigations reported in the literature. Griyns (9) found that chicken and horse erythrocytes remained intact in isotonic solutions of mannitol and inositol. Hedin (11) reported that mannitol and adonitol penetrated ox erythrocytes only slightly and erythritol somewhat more rapidly. Human erythrocytes were found to be impermeable to mannitol and dulcitol and slightly permeable to adonitol (12). Five and six carbon alcohols did not perceptibly penetrate human erythrocytes (13).

TABLE III.—VALUES FOR *i* FOR VARIOUS POLYHYDRIC ALCOHOLS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

| Compound | Hemolysis, % 25 | 50 | 75 | Average |
|---|---|---|---|---|
| Mannitol | 1.26 | 1.29 | 1.33 | 1.29 |
| Sorbitol | 1.23 | 1.28 | 1.33 | 1.28 |
| Inositol[b] | 1.27 | 1.30 | 1.35 | 1.31 |
| Mannitol[c] | 1.23 | 1.24 | 1.26 | 1.24 |
| Sorbitol[c] | 1.22 | 1.24 | 1.28 | 1.25 |
| Inositol[c] | 1.18 | 1.22 | 1.26 | 1.22 |

[a] Unless otherwise indicated, all *i* values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In the presence of 0.2% sodium chloride.

TABLE IV.—VALUES FOR *i* FOR VARIOUS POLYHYDRIC ALCOHOLS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

| Compound | Hemolysis, % 25 | 50 | 75 | Average |
|---|---|---|---|---|
| Erythritol | 0.47 | 0.53 | 0.60 | 0.53 |
| Pentaerythritol | 0.61 | 0.66 | 0.72 | 0.66 |
| Mannitol | 1.32 | 1.37 | 1.42 | 1.37 |
| Sorbitol | 1.31 | 1.37 | 1.41 | 1.36 |
| Inositol[b] | 1.35 | 1.40 | 1.46 | 1.40 |
| Mannitol[c] | 1.20 | 1.21 | 1.25 | 1.22 |
| Sorbitol[c] | 1.19 | 1.21 | 1.24 | 1.21 |
| Inositol[c] | 1.20 | 1.24 | 1.30 | 1.25 |

[a] Unless otherwise indicated, all *i* values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

Values for *i* of approximately 1.20 for amino acids and sugars using rabbit erythrocytes were reported by Grosicki and Husa (2). They found similar *i* values for dextrose, sucrose, and lactose, also with rabbit erythrocytes. Reference to Tables III and IV shows *i* values for mannitol, sorbitol, and inositol to be approximately 1.40 with human erythrocytes and 1.30 with rabbit erythrocytes. These values might be interpreted as showing 30 to 40% dissociation of these polyhydric alcohols, but this would be contrary to the recognized chemical properties of such compounds. In solutions of some substances erythrocytes lose electrolytes from within the cell, thus causing a decrease in the internal osmotic pressure and a corresponding increase in the resistance to hemolysis. Grosicki and Husa (2) hypothesized that such exosmosis from erythrocytes might have occurred in the solutions of amino acids and sugars they studied. Electrolyte loss would cause the cells to be in equilibrium with lower concentrations of the amino acids and sugars than would otherwise be the case, and the *i* values would be higher than if no

electrolyte loss had occurred. Thus, the fact that the $i$ values for mannitol, sorbitol, and inositol were found to be greater than 1.00 might be ascribed to loss of electrolytes from within the erythrocytes.

Hober (14) stated that the severe alteration of the normal permeability of erythrocytes caused by isotonic solutions of some nonelectrolytes could be largely reduced by the addition of small proportions of an electrolyte such as sodium chloride. Reference to Tables III and IV reveals the effects of 0.2% sodium chloride upon the $i$ values of mannitol, sorbitol, and inositol in rabbit and human blood. Using rabbit erythrocytes there appeared to be little difference in the $i$ values determined in the presence of 0.2% sodium chloride and the $i$ values determined with the alcohols alone. With human erythrocytes the $i$ values seem to be slightly lower in the presence of 0.2% sodium chloride. However, even after the addition of 0.2% sodium chloride, these $i$ values are still greater than 1.00. This situation is similar to that reported by Grosicki and Husa (2) for the sugars they investigated using rabbit erythrocytes; even in the presence of 0.2% sodium chloride the $i$ values of the sugars remained approximately 1.20. They stated that the presence of sodium chloride apparently did not correct the abnormal exosmosis. With mannitol, sorbitol, and inositol the presence of of 0.2% sodium chloride apparently also did not alter abnormal exosmosis.

Using human erythrocytes, Grosicki and Husa (2) reported an average $i$ value of 1.17 for dextrose. In the presence of 0.2% sodium chloride, dextrose gave an $i$ value of 0.66. In the present investigation pentaerythritol alone was found to have an average $i$ value of 0.66 and erythritol 0.53, when human erythrocytes were employed. Such low $i$ values might indicate a degree of permeability of the erythrocytes by the compounds in question.

**Structure and Activity.**—The monohydric alcohols investigated which contained from one to four carbon atoms did not prevent hemolysis. The difficulty in clearly relating hemolytic effects and chemical structure of the straight chain alcohols was noted by Ponder (15). Fuhner and Neubauer (16) reported that the hemolytic action of alcohols increased with increasing molecular weight. Ponder and Hyman (17) found that primary alcohols of three to nine carbon atoms showed a logarithmic relationship between the number of carbon atoms and the power to accelerate the hemolytic effect of saponin solutions. Alcohols containing three or less carbon atoms were reported to rapidly hemolyze dog erythrocytes (18).

Various isomerically related monohydric alcohols were included in the present investigation. There appeared to be little difference between the hemolytic effects of *n*-propyl and isopropyl alcohols and *n*-butyl, isobutyl, *sec*-butyl, and *tert*-butyl alcohols even in the presence of sodium chloride. However, branch chain alcohols were reported by Seelich and Pirquit (19), to possess weaker hemolytic effects than the corresponding normal alcohols; the results in Table II are in accord with this statement, since *n*-butyl alcohol in the presence of 0.6% sodium chloride caused 100% hemolysis at a lower concentration than the branched chain isomers.

Several polyhydric alcohols (ethylene glycol, 1,2-propanediol, glycerol, and 1,3-butanediol) did not show the ability to prevent hemolysis. Furthermore, in the presence of 0.2% sodium chloride no preventive tendencies were found. However, in the presence of 0.6% sodium chloride no hemolysis was noted.

Jacobs and co-workers (20) found that each successive hydroxyl group added to the propane molecule decreased the rate of penetration of ox and rabbit erythrocytes. In the diols, the position of the hydroxyl groups seemed important, for penetration occurred more rapidly when these groups were on adjacent carbon atoms than when they were located at the ends of the molecule. In the present investigation, there seemed to be no difference in the hemolytic activities of *n*-propyl alcohol, isopropyl alcohol, 1,2-propanediol, and glycerol when these were used alone or in the presence of 0.2% sodium chloride. However, when 0.6% sodium chloride was employed, glycerol and 1,2-propanediol did not cause hemolysis, whereas *n*-propyl and isopropyl alcohols caused hemolysis. A similar situation was found in the case of the four-carbon alcohols investigated. None of the isomeric butyl alcohols nor 1,3-butanediol prevented hemolysis when used alone or in the presence of 0.2% sodium chloride. When 0.6% sodium chloride was used, 1,3-butanediol did not cause hemolysis, whereas the isomeric butyl alcohols did cause hemolysis. Erythritol, furthermore, was found to prevent hemolysis, having an $i$ value of approximately 0.55.

It was possible to obtain $i$ values for the five-carbon alcohol, pentaerythritol, and the six-carbon alcohols, sorbitol, mannitol, and inositol, indicating that these compounds were able to prevent hemolysis. Hedin (11) observed that the rate of penetration of polyhydric alcohols decreased with the number of hydroxyl groups present. Erythritol was reported (18) as causing a slower hemolysis of dog erythrocytes than alcohols of three or less carbon atoms; five- and six-carbon alcohols were found to hemolyze the blood samples very slowly.

## SUMMARY

1. The degree of hemolysis of rabbit and human erythrocytes in solutions of monohydric and polyhydric alcohols of varying concentration was determined quantitatively and compared with that occurring in various concentrations of sodium chloride. When possible, these data were used to calculate van't Hoff $i$ values.

2. Approximately 100 per cent hemolysis occurred, at concentrations calculated to be isosmotic, with all of the alcohols investigated which had a molecular weight less than that of erythritol; those of greater molecular weight prevented hemolysis.

3. The alcohols of lower molecular weight were found also to cause complete hemolysis in the presence of 0.2 per cent sodium chloride. Of these, the monohydric alcohols, when present in lower concentrations, did not cause hemolysis in the presence of 0.6 per cent sodium chloride; in higher concentrations, however, except for methyl alcohol, the monohydric alcohols caused

hemolysis in the presence of 0.6 per cent sodium chloride. This was true of the normal monohydric alcohols investigated as well as their isomers.

4. The low molecular weight diols and triol investigated did not cause hemolysis in the presence of 0.6 per cent sodium chloride.

5. For erythritol and pentaerythritol *i* values of less than 1.00 were found, possibly indicating a degree of permeability of the erythrocytes by these compounds.

6. Mannitol, sorbitol, and inositol showed *i* values of approximately 1.40 with human blood and 1.30 with rabbit blood. Values for *i* which are greater than 1.00 might be ascribed to a loss of electrolytes from within the erythrocytes.

7. It appeared that the phenomena occurring at the erythrocyte membrane in the case of alcohols might be influenced by molecular weight and the number of hydroxyl groups present. When an increase in molecular weight was due to hydroxyl groups, the degree of hemolysis occurring seemed to decrease. Polyhydric compounds of four or more hydroxyl groups and four or more carbon atoms prevented hemolysis. Thus, the ratio of hydroxyl groups to carbon atoms present in the molecule might be important.

8. The results indicated that the *i* values for sodium chloride obtained at 37° were directly comparable with the data obtained at 25° by previous workers.

## REFERENCES


(1) Husa, W. J., and Adams, J. R., THIS JOURNAL, **33**, 329(1944).
(2) Grosicki, T. S., and Husa, W. J., *ibid.*, **43**, 632(1954).
(3) Easterly, W. D., and Husa, W. J., *ibid.*, **43**, 753(1954).
(4) Hartman, C. W., and Husa, W. J., *ibid.*, **46**, 430 (1957).
(5) Cadwallader, D. E., and Husa, W. J., *ibid.*, **47**, 705 (1958).
(6) Thomasson, C. L., and Husa, W. J., *ibid.*, **47**, 711 (1958).
(7) Hunter, F. T., *J. Clin. Invest.*, **19**, 691(1940).
(8) Harned, H. S., and Owen, B. B., "The Physical Chemistry of Electrolyte Solutions," 3rd ed., Reinhold Publishing Corp., New York, N. Y., 1958, p. 492.
(9) Griyns, G., *Pflüger's Arch. ges. Physiol.*, **63**, 86 (1896); cited by Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," 1st ed., MacMillan Co., New York, N. Y. 1943, p. 86.
(10) Booij, H. L., and Hijer, J., and Dijkshoorn, W., *Acta Physiol. et Pharmacol. Neerl.*, **1**, 617(1950).
(11) Hedin, S., *Pflüger's Arch. ges. Physiol.*, **68**, 229(1897); cited by Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," 1st ed., MacMillan Co., New York, N. Y., 1943, p. 86.
(12) Kozawa, S., *Biochem. Z.*, **60**, 231(1914).
(13) LeFevre, P. G., and Davies, R. I., *J. Gen. Physiol.*, **34**, 515(1951).
(14) Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., The Blakiston Co., Philadelphia, Pa., 1945, p. 238.
(15) Ponder, E., "Hemolysis and Related Phenomena," Grune and Stratton, New York, N. Y., 1948, pp. 280–282.
(16) Fuhner, H., and Neubauer, E., *Naunyn-Schmiedeberg's Arch. Expil. Pathol. Pharmakol.*, **56**, 333(1907).
(17) Ponder, E., and Hyman, C., *Proc. Soc. Expil. Biol. Med.*, **42**, 320(1939).
(18) Kunkel, A. M., Carr, C. J., and Krantz, J. C., *ibid.*, **42**, 438(1939).
(19) Seelich, F., and Pirquit, A., *Z. Immunitätsforsch.*, **86**, 358(1935); through *Chem. Abstr.*, **30**, 3066(1936).
(20) Jacobs, M. H., Glassman, H. N., and Parpart, A. K., *J. Cellular Comp. Physiol.*, **7**, 197(1935).


# Isotonic Solutions X*

## The Permeability of Red Corpuscles to Various Local Anesthetics

By DAVID MARCUS† and WILLIAM J. HUSA

**In the present investigation, the effects of local anesthetics in preventing hemolysis of human and rabbit erythrocytes were studied. Employing the hemolytic method, van't Hoff *i* values were obtained for several local anesthetics. The results indicate that these compounds have lower *i* values than might be expected. The addition of 0.2 per cent sodium chloride depressed the *i* values. Hemolytic *i* values were not determinable for the majority of the compounds studied; these compounds caused hemolysis even in the presence of 0.6 per cent sodium chloride.**

THE ADVANTAGE of applying the hemolytic method to the preparation of isotonic solutions was pointed out by the investigations of Husa and co-workers (1–8). They emphasized that the osmotic effect of a substance upon an erythrocyte membrane depends not only upon the number of particles contributed by the solute but also upon whether or not they penetrate the membrane or liberate hemoglobin in some other manner. If the compound under consideration does not exhibit the *i* value expected on the basis of the number of ions it is theoretically presumed to contribute, then extra deliberation is indicated in the preparation of isotonic solutions of the substance. These observations make it apparent that calculation of tonicity, based solely upon colligative properties, may prove to be inaccurate

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when applied to the preparation of solutions isotonic with the blood.

In the present investigation, an inquiry was made into the effects of local anesthetics in preventing hemolysis of human and rabbit blood. An examination of the literature revealed only two references (9, 10) relating to the effect of local anesthetics upon the red corpuscle; the majority of the compounds studied in this investigation had not been previously reported with reference to their effect upon erythrocytes. The hemolytic *i* values for several anesthetics were determined. Experiments were also conducted to determine the effect that the local anesthetics have in preventing hemolysis when used in the presence of 0.2 per cent sodium chloride. It has been previously reported (11) that the addition of small proportions of an electrolyte, such as sodium chloride, reduces the severe alteration of permeability that occurs when erythrocytes are placed in solutions of some nonelectrolytes. Hartman and Husa (4) observed that this is also true in solutions of some electrolytes. Determinations were similarly conducted in the presence of 0.6 per cent sodium chloride; the purpose of these experiments was to evaluate the effect of these compounds upon red corpuscles even when the salt concentration was sufficient to prevent osmotic hemolysis if no other substance were present.

## EXPERIMENTAL

**Collection of Blood.**—The blood used in the present investigation was obtained in the same manner as described previously by Husa and coworkers (2, 3). The human blood samples were obtained from the veins of the arms of a twenty-seven-year-old male. Several experiments were carried out employing blood from other donors.

**Preparation of Solutions.**—The solutions used in this study were prepared in essentially the same manner as described by other workers (2, 3).

**Quantitative Determination of Per Cent Hemolysis.**—The calculation of hemolytic *i* values has been described in detail by Grosicki and Husa and Easterly and Husa (2, 3).

## DISCUSSION

**Variability of Blood.**—A range of concentrations of sodium chloride solutions was employed for each blood sample. Thus, any differences in fragility were compensated for by comparison of the degree of hemolysis occurring in solutions of the substance under investigation with the degree of hemolysis in various concentrations of sodium chloride. Duplicate tubes of all solutions were employed within each experiment. Five separate tubes were used for each blood sample for the 100% hemolysis readings; two tubes were placed at the beginning and three at the end of the experimental run. For each local anesthetic studied experiments were conducted using at least two different blood samples of the same species.

**Chemicals.**—Samples of the local anesthetics employed in this investigation were generously contributed by their manufacturers: butacaine sulfate, procaine hydrochloride, and pramoxine hydrochloride by Abbott Laboratories; lidocaine hydrochloride by Astra Pharmaceutical Products, Inc.; dibucaine hydrochloride by Ciba Pharmaceutical Products, Inc.; cyclomethycaine hydrochloride and piperocaine hydrochloride by Eli Lilly and Co.; diperodon hydrochloride by the Wm. S. Merrell Co.; hexylcaine hydrochloride by Merck Sharp & Dohme Research Laboratories; intracaine hydrochloride by Philadelphia Ampoule Labs.; procaine isobutyrate by William H. Rorer, Inc.; benoxinate hydrochloride by Smith-Dorsey; procaine amide hydrochloride by E. R. Squibb & Sons; 2-chloroprocaine hydrochloride by Wallace & Tiernan Inc.; phenacaine hydrochloride, 2-propoxyprocaine hydrochloride, and tetracaine hydrochloride by Sterling-Winthrop Research Institute.

**Results with Procaine Hydrochloride, Procaine Isobutyrate, and Procaine Amide Hydrochloride.**—Procaine hydrochloride apparently exhibited some osmotic effect in preventing hemolysis in human and rabbit blood. Hemolytic *i* values were determinable only for 75% hemolysis of human blood and for 50% and 75% hemolysis of rabbit blood. There are two opposing variables. The first is the decrease in hemolysis due to an increase in the number of particles in solution (increasing concentration). The second is the increase in hemolysis due to the effect of increasing concentration of the compound upon the corpuscular membrane. The curve for per cent hemolysis *versus* concentration is therefore the composite curve for these two variables. There were several instances where hemolytic *i* values were only obtainable from 75% or 50% and 75% hemolysis values (see Tables I and II). This was due to the effect of the anesthetic upon the red cell membrane in preventing the development of a normal sigmoid curve, i.e., a curve from which 25, 50, and 75% hemolysis values are obtainable. Procaine hydrochloride solutions caused a significant proportion of hemolysis in the presence of 0.6% sodium chloride. Thus the local anesthetic apparently had an appreciable effect upon the corpuscular membrane. The average hemolytic *i* value for procaine hydrochloride, in human blood at 75% hemolysis was 0.57; in rabbit blood, at 50% and 75% hemolysis, the *i* value was 0.49.

The presence of 0.2% sodium chloride has been reported to restore the normal permeability of erythrocytes in some instances (2, 4, 6, 11). The average value of *i* for procaine hydrochloride, in the presence of 0.2% sodium chloride, was obtainable only for 75% hemolysis of both human and rabbit blood; these values were 0.30 and 0.28, respectively. It has been reported that the addition of sodium chloride to a solution of a nitrogenous basic hydrochloride represses the dissociation of the nitrogenous base so markedly that it behaves almost like a nonelectrolyte (12). The degree of repression of dissociation is determined by the basic strength of the nitrogenous base. Thus the number of osmotically active particles is dependent upon the concentra-

TABLE I.—VALUES OF *i* FOR VARIOUS LOCAL ANESTHETICS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF HUMAN ERYTHROCYTES[a]

| Anesthetic Hydrochloride | —Hemolysis, %— 25 | 50 | 75 | Average |
|---|---|---|---|---|
| Procaine[b] | ... | ... | 0.57 | 0.57 |
| Procaine[c] | ... | ... | 0.30 | 0.30 |
| Procaine amide[b] | 0.37 | 0.44 | 0.50 | 0.43 |
| Procaine amide[b, c] | ... | 0.27 | 0.28 | 0.28 |
| 2-Propoxyprocaine | 0.81 | 1.27 | 1.67 | 1.25 |
| 2-Chloroprocaine | ... | ... | 1.42 | 1.42 |
| 2-Chloroprocaine[c] | ... | ... | 0.77 | 0.77 |

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

TABLE II.—VALUES OF *i* FOR VARIOUS LOCAL ANESTHETICS, CALCULATED FROM CONCENTRATIONS CAUSING 25, 50, AND 75% HEMOLYSIS OF RABBIT ERYTHROCYTES[a]

| Anesthetic Hydrochloride | —Hemolysis, %— 25 | 50 | 75 | Average |
|---|---|---|---|---|
| Procaine[b] | ... | 0.46 | 0.52 | 0.49 |
| Procaine[c] | ... | ... | 0.28 | 0.28 |
| Procaine amide[b] | ... | ... | 0.42 | 0.42 |
| Procaine amide[c] | ... | ... | 0.26 | 0.26 |
| 2-Propoxyprocaine | 0.79 | 0.94 | 1.12 | 0.95 |
| 2-Chloroprocaine | ... | ... | 0.68 | 0.68 |
| 2-Chloroprocaine[c] | ... | ... | 0.31 | 0.31 |

[a] Unless otherwise indicated all *i* values represent an average of two blood samples.
[b] Average of three blood samples.
[c] In presence of 0.2% sodium chloride.

tion of the basic hydrochloride and sodium chloride as well as the character of the basic hydrochloride (12). The lower *i* values obtained in the presence of sodium chloride might be ascribed to the repression of the dissociation of procaine hydrochloride by the salt. Thomasson (6) investigated the effect of 0.2% sodium chloride upon the hemolytic *i* values of various alkaloids. He found that the addition of sodium chloride lowered the *i* values of atropine sulfate, ephedrine hydrochloride and sulfate, and homatropine methylbromide.

Procaine isobutyrate failed to show any appreciable prevention of hemolysis at concentrations that were calculated to be isosmotic. The difference in permeability between procaine hydrochloride and procaine isobutyrate may be explained on the basis of the pH of these compounds. A 1% solution of the hydrochloride had a pH of 5.23 whereas the isobutyrate had a pH of 7.05. Goyan and Daniels (13) presented the equilibria involved in a solution of a procaine salt and stated that in general, neutral or slightly alkaline solutions favor a high equilibrium concentration of the free base. There have been many references to substantiate the premise that the free bases of weak electrolytes are able to penetrate the cell membrane while the cation cannot (14–17). As a consequence, it is to be expected that the isobutyrate would more easily penetrate the red cell than the hydrochloride due to the higher concentration of the free base, i. e., procaine isobutyrate would exhibit little or no appreciable osmotic effect.

The average hemolytic *i* value for procaine amide in human blood was 0.43 and in rabbit blood, at 75% hemolysis, was 0.42. The determinations involving human blood provided characteristically sigmoid curves; however, the per cent hemolysis could not be brought to zero. Instead, the curve indicated increased hemolysis at 10% (0.37 *M*) concentration of anesthetic. A possible explanation for this phenomenon would be the increased fragility of the erythrocytes noted at 10% concentration of anesthetic in the presence of 0.6% sodium chloride. Since hemolysis occurred to some extent throughout the range of concentrations employed, the low *i* values could be attributed to this cause, i.e., the hemolysis due to the alteration of the membrane would release hemoglobin in addition to that already liberated from osmotic hemolysis. Rabbit cells were more sensitive to procaine amide and thus 25% and 50% hemolysis values were unobtainable.

The addition of 0.2% sodium chloride to procaine amide solutions lowered the hemolytic *i* values to 0.28 in human blood and to 0.26 in rabbit blood.

**Results With 2-Propoxyprocaine, 2-Chloroprocaine, and Benoxinate Hydrochlorides.**—The hemolytic *i* values for 2-propoxyprocaine hydrochloride were 1.25 in human blood and 0.95 in rabbit blood. Determinations were carried out in the presence of 0.6% sodium chloride and no significant degree of hemolysis was noted at the concentrations utilized in the calculation of hemolytic *i* values.

The hemolytic *i* values for 2-chloroprocaine hydrochloride were obtainable only for 75% hemolysis; the average value in human blood was 1.42, for rabbit blood 0.68. The addition of 0.2% sodium chloride reduced these values to 0.77 and 0.31, respectively. At the concentrations employed in the calculations of the *i* values, in both human and rabbit blood, approximately 50% hemolysis was observed in the presence of 0.6% sodium chloride. These results indicate that 2-chloroprocaine had an appreciable effect upon the erythrocyte membrane. Bodansky (18) reported that the halogen derivatives of fatty acids were much more lytic than the acids themselves, probably because they were much more highly dissociated and more soluble in lipids. Hober (19) stated that the introduction of polar groups decreased the penetrating capacity of a substance, whereas that of nonpolar groups, such as halogens, increased the penetrating capacity. The data obtained for 2-chloroprocaine apparently does not concur with the aforementioned contention that halogen substitution increases the penetrating capacity of a compound; the hemolytic *i* values for 2-chloroprocaine were higher than those of procaine. Paradoxically, however, hemolysis occurred at a lower concentration in the presence of 0.6% sodium chloride in solutions of the chlorine-substituted compound (see Table III). This evidence suggests that while the halogenated procaine does not penetrate the erythrocyte as readily as does procaine, it has a more profound effect upon the membrane.

Experiments with benoxinate hydrochloride demonstrated that this compound had no apparent effect in preventing hemolysis. A concentration of 0.42% ($1.2 \times 10^{-3}$ *M*) benoxinate hydrochloride caused 50% hemolysis of human blood in the presence of 0.6% sodium chloride; in rabbit blood, the concentration was 0.16% ($4.6 \times 10^{-3}$ *M*). It is of interest to note that 2-propoxyprocaine and benoxinate differ in that the latter is a 3-*n*-butoxypro-

caine. It seems as if the substitution of the propoxy group with a butoxy group is responsible for the vastly greater hemolytic effect of benoxinate hydrochloride.

A partial explanation for the unusually low hemolytic $i$ values obtained for procaine, procaine amide, 2-propoxyprocaine, and 2-chloroprocaine hydrochlorides may lie in a consideration of the possible formation of micellular aggregates by these compounds. Debye (20, 21) reported that substituted quaternary ammonium salts and amine hydrochlorides often form micellular aggregates in solution. Hammarlund and Pedersen-Bjergaard (22) indicated that solutions of dibucaine (0.07 $M$), tetracaine (0.13 $M$), and pramoxine (0.08 $M$) hydrochlorides behaved in a manner suggesting the formation of micelles. The formation of such aggregates would produce fewer particles in solution than expected from the original concentration and would consequently result in lower $i$ values. The addition of any substance which would disturb the micellular formation by either increasing or decreasing the number of particles would alter the osmotic effect; 0.2% sodium chloride might act in such a manner. The formation of micelles by these compounds has not been experimentally established; however, the substituted procaine compounds are structurally similar to tetracaine, are amine hydrochlorides and would therefore be expected to behave similarly to some extent. In any event, this concept would provide only a partial explanation for the low $i$ values; the data from hemolytic determinations in 0.6% sodium chloride indicated that these anesthetics caused some hemolysis regardless of osmotic pressure.

**Results with Esters of Benzoic and Para-aminobenzoic Acid in the Presence of 0.6% Sodium Chloride.**—The anesthetics of this group that were investigated were cyclomethycaine, hexylcaine, intracaine, piperocaine, and tetracaine hydrochlorides, and butacaine sulfate. Preliminary hemolytic determinations disclosed that these compounds had no apparent effect in inhibiting hemolysis in concentrations that were supposedly isotonic according to physicochemical data. In compounds of this general structure, the alteration or addition of groups may produce a profound effect upon their properties as regards hemolysis. For example, piperocaine and cyclomethycaine differ only in the presence of a $p$-cyclohexyloxy group on the benzoic acid moiety of the latter compound. The addition of this substituent group is apparently responsible for the vastly greater hemolytic properties of cyclomethycaine. The concentrations of local anesthetic causing 50% hemolysis of erythrocytes in 0.6% sodium chloride is presented in Tables III and IV. In human blood, the hemolytic activity increased in the following order: intracaine, piperocaine, hexylcaine, tetracaine, butacaine, and cyclomethycaine. In rabbit blood the order was the same with the exception that hexylcaine precedes piperocaine. Cyclomethycaine caused hemolysis at the lowest concentration of all the compounds examined.

**Results with Anesthetics of Heterogeneous Structure in the Presence of 0.6% Sodium Chloride.**—A study was made of diethylaminoethanol, dimethylaminoethanol, and dibucaine, diperodon, lidocaine, phenacaine, and pramoxine hydrochlorides.

TABLE III.—CONCENTRATIONS OF LOCAL ANESTHETIC CAUSING 50% HEMOLYSIS OF HUMAN ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE[a]

| Local Anesthetic[b] | Molarity | Per Cent |
|---|---|---|
| Procaine amide | ...[c] | ... |
| 2-Propoxyprocaine | ...[c] | ... |
| Procaine | $3.1 \times 10^{-1}$ | 8.6 |
| Procaine isobutyrate | $2.0 \times 10^{-1}$ | 6.9 |
| 2-Chloroprocaine | $1.6 \times 10^{-1}$ | 5.1 |
| Lidocaine | $1.5 \times 10^{-1}$ | 4.2 |
| Intracaine | $1.1 \times 10^{-1}$ | 3.2 |
| Piperocaine | $8.5 \times 10^{-2}$ | 2.5 |
| Hexylcaine | $2.9 \times 10^{-2}$ | 0.87 |
| Phenacaine | $1.4 \times 10^{-2}$ | 0.49 |
| Benoxinate | $1.2 \times 10^{-2}$ | 0.42 |
| Dimethylaminoethanol[d] | $1.1 \times 10^{-2}$ | 0.10 |
| Tetracaine | $9.2 \times 10^{-3}$ | 0.28 |
| Pramoxine | $6.1 \times 10^{-3}$ | 0.2 |
| Butacaine sulfate | $5.6 \times 10^{-3}$ | 0.40 |
| Diethylaminoethanol[d] | $4.1 \times 10^{-3}$ | 0.05 |
| Diperodon | $2.6 \times 10^{-3}$ | 0.11 |
| Dibucaine | $1.8 \times 10^{-3}$ | 0.07 |
| Cyclomethycaine | $5.5 \times 10^{-4}$ | 0.02 |

[a] Values obtained by interpolation from curves of per cent hemolysis *versus* concentration; values represent average of two blood samples.
[b] Anesthetic as the hydrochloride unless otherwise indicated.
[c] These compounds did not cause 50% hemolysis.
[d] As the alcohol.

TABLE IV.—CONCENTRATION OF LOCAL ANESTHETIC CAUSING 50% HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF 0.6% SODIUM CHLORIDE[a]

| Local Anesthetic[b] | Molarity | Per Cent |
|---|---|---|
| Procaine | ...[c] | ... |
| Intracaine | ...[c] | ... |
| Procaine isobutyrate | $8.5 \times 10^{-2}$ | 2.8 |
| Procaine amide | $8.1 \times 10^{-2}$ | 2.2 |
| 2-Propoxyprocaine | $3.9 \times 10^{-2}$ | 1.3 |
| 2-Chloroprocaine | $2.7 \times 10^{-2}$ | 0.83 |
| Pramoxine | $2.1 \times 10^{-2}$ | 0.69 |
| Lidocaine | $2.0 \times 10^{-2}$ | 0.53 |
| Hexylcaine | $1.7 \times 10^{-2}$ | 0.52 |
| Piperocaine | $9.9 \times 10^{-3}$ | 0.29 |
| Phenacaine | $8.8 \times 10^{-3}$ | 0.31 |
| Benoxinate | $4.6 \times 10^{-3}$ | 0.16 |
| Tetracaine | $4.4 \times 10^{-3}$ | 0.13 |
| Dimethylaminoethanol[d] | $3.6 \times 10^{-3}$ | 0.03 |
| Butacaine Sulfate | $3.4 \times 10^{-3}$ | 0.24 |
| Diperodon | $3.3 \times 10^{-3}$ | 0.14 |
| Diethylaminoethanol[d] | $1.8 \times 10^{-3}$ | 0.02 |
| Dibucaine | $8.9 \times 10^{-4}$ | 0.034 |
| Cyclomethycaine | $3.8 \times 10^{-4}$ | 0.015 |

[a] Values obtained by interpolation from curves of per cent hemolysis *versus* concentration; values represent average of two blood samples.
[b] Anesthetic as the hydrochloride unless otherwise indicated.
[c] These compounds did not cause 50% hemolysis.
[d] As the alcohol.

These compounds also did not prevent osmotic hemolysis in concentrations that were presumably isotonic. Several investigators (23, 24) have reported that procaine was hydrolyzed by serum to diethylaminoethanol and $p$-aminobenzoic acid. Dimethylaminoethanol would be the amino alcohol resulting from the hydrolysis of tetracaine. The pH of these two amino alcohols was 10.1 at the concentration causing 50% hemolysis in the presence of 0.6% sodium chloride (see Tables III and IV). The

excessive alkalinity is apparently responsible for the hemolytic effect of these compounds.

The concentrations of dibucaine, diperodon, lidocaine, phenacaine, and pramoxine hydrochlorides causing 50% hemolysis in the presence of sodium chloride are listed in Tables III and IV. Hemolytic effect increased in the following order: lidocaine, phenacaine, pramoxine, diperodon, and dibucaine; in rabbit blood the order was pramoxine, lidocaine, phenacaine, diperodon, and dibucaine.

The effect of micelle formation by dibucaine, tetracaine, and pramoxine hydrochlorides, described by Hammarlund and Pedersen-Bjergaard (22), was not reflected in the data obtained in this investigation. The explanation for this would seem to lie in the fact that the concentrations of anesthetic used in the hemolytic determinations were considerably lower than those described as forming micellular aggregates.

**Penetration of the Free Base.**—Jacobs (25, 26) made the first analysis of the behavior of the ammonium salts of weak acids. In this case, both anion and cation can penetrate in the unionized form. Jacobs came to the conclusion that $NH_3$, formed by the hydrolysis, is the form in which ammonia penetrates the cell membrane and found that in the case of red cells, hemolysis occurred with $NH_4$ salts of even strong acids. This is because red cells are normally permeable to anions, so that in the case of $NH_4Cl$, ammonia penetrates as $NH_3$ and the chloride as the ion, so that there is no impermeable substance to prevent the red cells from swelling and bursting. If the local anesthetics may be considered as $(R_2NH)Cl$, it is possible to make an analogy with $NH_4Cl$. The free base $R_2N$ may then be considered to enter the cell as does $NH_3$. Husa and Adams (1) found that $NH_4Cl$ was hemolytic even in the presence of 0.6% sodium chloride. They concluded that osmotic pressure considerations alone appear insufficient to explain these results; apparently $NH_4Cl$ brings about the release of hemoglobin by some other mechanism, perhaps by dissolving some membrane constituent and thus altering its permeability. This explanation may in part explain the hemolytic effects of many of the local anesthetics employed in this investigation.

**Effect of Cholinesterase Activity.**—Several investigators have established that a relationship exists between the permeability of erythrocytes and cholinesterase activity (27, 28, 29). The consensus seems to be that the permeability of red cells would be altered by inhibition of cholinesterase in the cell membrane. It has also been confirmed that local anesthetics are inhibitory to cholinesterase; indeed procainesterase was found to be identical with cholinesterase (30–36). A logical assumption would be that the local anesthetics influence erythrocyte permeability to some extent by the inactivation of cholinesterase. Alteration of the cell membrane by inhibition of membrane cholinesterase may explain the release of hemoglobin from red corpuscles in solutions of local anesthetics containing 0.6% sodium chloride.

## SUMMARY

1. Hemolytic $i$ values have been determined for several local anesthetics but were not determinable for the majority of the compounds investigated.

2. It was found that the addition of 0.2% sodium chloride lowered the $i$ values.

3. Most of the local anesthetics studied caused hemolysis even in the presence of 0.6% sodium chloride.

4. In compounds of the benzoic and *p*-aminobenzoic ester type, the alteration or addition of groups produced a profound effect upon their properties as regards hemolysis.

## REFERENCES

(1) Husa, W. J., and Adams, J. R., THIS JOURNAL, **33**, 329(1944).
(2) Grosicki, T. S., and Husa, W. J., *ibid.*, **43**, 632(1954).
(3) Easterly, W. D., and Husa, W. J., *ibid.*, **43**, 750(1954).
(4) Hartman, C. W., and Husa, W. J., *ibid.*, **46**, 430(1957).
(5) Cadwallader, D. E., and Husa, W. J., *ibid.*, **47**, 705 (1958).
(6) Thomasson, L. C., and Husa, W. J., *ibid.*, **47**, 711 (1958).
(7) Ansel, H. C., and Husa, W. J., *ibid.*, **48**, 516(1959).
(8) Zanowiak, P., and Husa, W. J., *ibid.*, **48**, 565(1959).
(9) Gessner, O., Walther, M., and Reinhardt, K., *Naunyn-Schmiedberg's Arch. Exptl. Pathol. Pharmakol.*, **186**, 329(1937).
(10) Pribram, E., *Pflüger's Arch. ges. Physiol.*, **137**, 350(1911).
(11) Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., The Blakiston Co., Philadelphia, Pa., 1945, p. 238.
(12) "Pharmacists Reference," 8th rev., Eli Lilly and Co., Indianapolis, Ind., 1957, p. 241.
(13) Goyan, F. M., and Daniels, T. C., THIS JOURNAL, **30**, 98(1941).
(14) Goodman, L., and Gilman, A., "The Pharmacological Basis of Therapeutics," 2nd ed., The Macmillan Co., New York, N. Y., 1955, p. 357.
(15) Dawes, G. S., *Brit. J. Pharmacol.*, **1**, 90(1946).
(16) Krahl, M. E., Keltch, A. K., and Clowes, G. H. A., *J. Pharmacol. Exptl. Therap.*, **68**, 330(1940).
(17) Cowles, P. B., *Yale J. Biol. and Med.*, **14**, 599(1942); cited by Burger, A., "Medicinal Chemistry," vol. 2, Interscience Publishers Inc., New York, N. Y., 1951, p. 475.
(18) Bodansky, M., Unpublished observations; cited by Ponder, E., "Hemolysis and Related Phenomena," Grune & Stratton, New York, N. Y., 1948, p. 281.
(19) Hober, R., "Physical Chemistry of Cells and Tissues," 1st ed., The Blakiston Co., Philadelphia, Pa., 1945, p. 231.
(20) Debye, P., *Ann. N. Y. Acad. Sci.*, **51**, 575(1949).
(21) Debye, P., *J. Phys. & Colloid Chem.*, **53**, 1(1949).
(22) Hammarlund, E. R., and Pedersen-Bjergaard, K., *Dansk Tidsskr. Farm. Suppl.*, **2**, 107(1956).
(23) Brodie, B., *et al.*, *J. Pharmacol. Exptl. Therap.*, **94**, 359(1948).
(24) Hauschild, F., *Pharmazie*, **5**, 105(1950).
(25) Jacobs, M. H., *J. Gen. Physiol.*, **5**, 181(1922).
(26) Jacobs, M. H., *Am. J. Physiol.*, **68**, 134(1924).
(27) Grieg, M., and Holland, W. C., *Arch. Biochem.*, **23**, 370(1949).
(28) Holland, W. C., and Graham, J. H., *Am. J. Physiol.*, **183**, 538(1955).
(29) Lindvig, P. E., Greig, M. E., and Peterson, S. W., *Arch. Biochem.*, **30**, 241(1951).
(30) Kalow, W., *J. Pharmacol. Exptl. Therap.*, **104**, 122(1952).
(31) Ammon, I., and Zipk, K., *Klin. Wochschr.*, **20**, 696(1941).
(32) Ammon, I., and Zipk, K., *ibid.*, **20**, 1176(1941).
(33) Adriani, J., and Rosenstine, E., *Anesthesia & Analgesia*, **20**, 109(1941).
(34) Zeller, E., and Bisseger, A., *Helv. Chim. Acta*, **26**, 1619(1943).
(35) Adler, P., Gal, I., and Vegh, L., *Z. Vitamin-, Hormon-u. Fermentforsch.*, **3**, 236(1950); through *Chem. Abstr.*, **44**, 10913(1950).
(36) Bullock, K., *Quart. J. Pharm. and Pharmacol.*, **21**, 266(1948).

# A Study of Permeability to Water Vapor of Fats, Waxes, and Other Enteric Coating Materials*

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**Apparent diffusivity and permeability of a variety of materials commonly employed to delay drug release from certain dosage forms have been evaluated using the lag time method. Several cellulose and glyceryl esters were studied and their permeability shown to vary with the relative polarity of these compounds. The influences of such variables as temperature, relative humidity, and compositions of mixtures were investigated. Thermal coefficients obtained for glyceryl monostearate suggest that the apparent overall heat of activation for permeation is about 8 Kcal./mole. The same system exhibits a very high dependency on relative humidity showing proportionally higher permeability near saturation than at lower values.**

INFORMATION relative to resistance toward permeation of various packaging and coating substances by aqueous vapor is of considerable importance to those concerned with formulation of pharmaceutical dosages. Since moisture pick-up or loss from drug preparations while in storage or on store shelves can often lead to undesirable results, materials highly resistant to moisture permeation are usually employed in pharmaceutical packaging. Many recent container materials, such as some plastics, exhibit rather low impedance to movement of water vapor. *Modus operandi* of some enteric and delayed-action dosage forms, on the other hand, appears to depend in part on controlled moisture penetration in regulating the rate of release of active medicaments.

Results of investigation of factors which influence the relative resistance of various plastics and coating materials toward penetration by water vapor are presented in this report. Although some new data on permeation of some plastics have been obtained, the primary aim of this communication is to present new information relative to the permeability of various coating and enteric materials used with or over compressed tablets and related dosage forms to produce delayed or sustained action effect. The influences of composition, temperature, and relative humidity on the apparent diffusivity of and permeability to water molecules have been investigated.

## LAWS GOVERNING PERMEATION PROCESS

Based on an analogy of heat conduction through solid media, Fick, in 1855 was able to propose a general law for diffusion. The essence of Fick's law is that the driving force which causes the transfer of a substance from regions of high to regions of low concentration is proportional to the concentration gradient. Fick's law (1) is commonly written as

$$Q = -D\frac{dc}{dx} \qquad \text{(Eq. 1)}$$

where $Q$ is the flux or the amount of material crossing a plane of unit area per unit time, $dc/dx$ the concentration gradient, $D$ is the diffusion coefficient and has the dimensions of cm.$^2$ sec.$^{-1}$. In this work $Q$ has been expressed as cc. of gas at normal temperature and pressure. The negative sign in Fick's law indicates that flow is directed toward the area of decreasing concentration.

The diffusion coefficient is a measure of the amount of material which would diffuse across a unit area under a unit concentration gradient in unit time. The application (2) of Fick's first law for diffusion requires the determination of the flux $Q$ and the concentration gradient. The fact that the law specifies conditions of a constant concentration gradient implies the establishment of a steady state.

The assumption that $D$ is independent of the concentration is not always valid. For exact analysis it becomes necessary to treat $D$ as a variable in deriving an expression for the change of concentration with time. Treating $D$ as a variable, Fick's second law can be written as

$$\frac{dc}{dt} = \frac{d}{dx}\left(D\frac{dc}{dx}\right) \qquad \text{(Eq. 2)}$$

The concentration of the diffusing substance within the surface of a membrane is not always given by the gas or the vapor pressure of the substance, for equilibrium at the interface membrane gas will not be established in such cases where the transition of the gas through the membrane is not a rapid process compared with the diffusion. In such cases an absolute determination of the diffusion constant is not possible and one makes use, therefore, of the concept of a "quasi-stationary state." In the quasi-stationary state neither the concentrations themselves nor the concentration gradient is exactly constant (i.e., independent of time).

The most important factor influencing the solubility of a gas is pressure (4). The quantitative connection between solubility and pressure is generally expressed as Henry's law, which can be stated as the cc. of gas that will dissolve ideally in

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one cc. of film material is proportional to the pressure of the gas with which it is in equilibrium. If $c$ is the cc. of the film material at equilibrium pressure $p$ then

$$c = Sp \qquad \text{(Eq. 3)}$$

where $S$ is a proportionality constant generally called the solubility coefficient or Henry's law constant and commonly defined in these connections as the cc. of gas that will dissolve ideally in one cc. of the film material when the pressure of the gas is one mm. of mercury.

If $h$ is the thickness of the film the change in concentration gradient can be written as

$$\frac{dc}{dx} = \frac{c}{h} \qquad \text{(Eq. 4)}$$

and from Eq. 3 the concentration gradient becomes

$$\frac{dc}{dx} = \frac{c}{h} = \frac{Sp}{h} \qquad \text{(Eq. 5)}$$

combining Eqs. 1 and 5 and rearranging

$$\frac{Q \times h}{p} = -DS \qquad \text{(Eq. 6)}$$

putting $P =$

$$\frac{Q \times h}{p}$$

one has

$$P = -DS \qquad \text{(Eq. 7)}$$

$P$ may be termed the permeability constant and may be defined as the cc. of gas at normal temperature and pressure that will pass per second through a unit area of the film one mm. thick when there is 1 cm. of mercury pressure between its faces.

Consequently the permeability of a film can be broken down into two contributing factors (3): the diffusion constant, which is a measure of the probability that each gas molecule will move in the direction of the concentration gradient, and the solubility coefficient, which measures the number of gas molecules per unit volume which contributes to this concentration gradient. The solubility coefficient according to Barrer (1) does not include the water molecules that are sorbed but only those that are ideally dissolved, and consequently this may or may not approximate the actual equilibrium solubility.

Anomalies observed in some of the present work, especially in the investigation of permeation through some of the waxes, are probably due to strong deviations from the assumption of a linear concentration gradient in the barrier phase, arising either from deviations from Henry's law of ideal solutions or from strong concentration dependency on the part of $D$. In an ideal steady-state concentration distribution across a membrane the drop in the concentration should be linear as shown in line A of Fig. 1, if the integral form of Fick's law is applicable. The concentration gradient in some of the waxes is, however, probably sigmoidal in character as shown in line B of Fig. 1, the gradient drop being small for the initial thickness and which then falls rapidly.

Henry's law, which can be shown to be a form of the general distribution law or of Raoult's law, is based on the ideal behavior of a gas, and any departure from ideality, such as those occurring with an easily compressible gas such as water vapor at low pressure or low temperature, will make the application of Henry's law invalid (5). Some causes of the departure from ideal behavior of a gas such as water vapor can be ascribed to self-association of the water molecules to form dimers or trimers in the barrier phase and diffusion as such, compound formation of the water with the material, and ionization of the vapor.

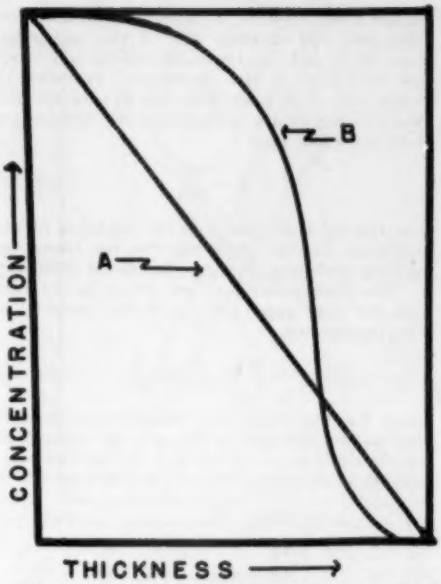


Fig. 1.—Hypothetical steady state concentration distribution across a membrane. A—linear. B—sigmoidal.

A suitable method of evaluating permeability, diffusivity, and solubility coefficients was developed by Barrer (6) based on a mathematical analysis by Daynes (7). A membrane of the polymer or other material is mounted in a diffusion cell and the permeation velocity is determined in the steady state as well as the rate at which that state is approached. The pressure on the ingoing side of the membrane is constant and is always much greater than that on the outgoing side.

The pressure on the outgoing side is plotted as a change in pressure *vs.* time curve. In most cases these curves emerge from the abscissa with a very small slope which continue over a period of time; the curve then gradually bends upwards and continues as a long straight line. There is an interval before the steady state can be approached due to finite diffusion velocity of the vapor within the membrane. Provided Fick's law is valid for the transport process, the intercept $L$ made by the asymptotic curve on the axis of time is

$$L = \frac{h^2 1}{D(C_1 - C_2)} \left( \frac{C_1}{6} + \frac{C_2}{3} - \frac{C_0}{2} \right) \qquad \text{(Eq. 8)}$$

for a membrane of thickness $h$, where $C_1$ and $C_2$

are the concentrations of the solute within the ingoing side and outgoing side of the membrane, respectively, and $C_0$ is the initial uniform concentration of solute in the membrane. Provided $C_0$ is zero and $C_2$ is much less than $C_1$ and also the diffusion coefficient is independent of concentration of gas in the film, then

$$L = \frac{h^2}{6D} \qquad \text{(Eq. 9)}$$

$L$ is the lag time, and $h$ is the thickness of the membrane in cm. Knowing the lag time, the diffusion coefficient $D$ can be calculated using Eq. 9. The permeability constant $P$ can be obtained from the slope $p$(mm.)/$t$(sec.) of the straight line using the equation

$$P = \frac{p}{t} \times \frac{V}{760} \times \frac{h}{pxa} \qquad \text{(Eq. 10)}$$

where $V$ is the volume into which the gas expands after passing through the film, $p$ is the vapor pressure of water in cm. of mercury on the high pressure side of the film, $h$ is the thickness of the film in cm., and $a$ is the area of the film in sq. cm. Using the relation $P = DS$ the solubility coefficient $S$ can be determined.

## EXPERIMENTAL

### Apparatus

The design of the all-glass apparatus used in the present study followed essentially that of Doty, *et al.* (8). The setup consisted of two similar halves as shown in Fig. 2, the left half in the diagram forming the high pressure or the water reservoir side and the right half the low pressure or the detection side. The two parts were connected in use by means of a standard taper joint (50/50). The barrier film was mounted in the male part of the joint effectively separating the two sides; this is shown in detail in Fig. 3.

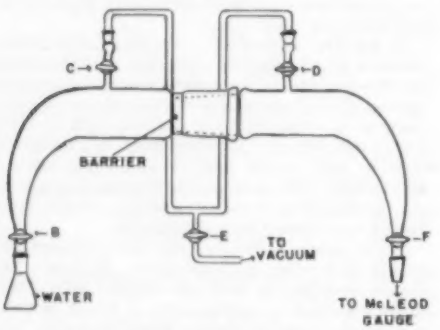


Fig. 2.—A diagram of the diffusion cell.

A McLeod gauge was used to measure the change in pressure on the low pressure side. With a condensable gas such as water vapor serious errors can result from condensation of the vapor on the bulb and capillary of the gauge on raising the mercury to take a reading. To avoid these errors the capillary of the gauge was jacketed with a water bath after the procedure of Doty, *et al.* (8).

The problem of temperature control during measurement was largely solved by placing the entire apparatus in a constant temperature room. The room was held to within ±0.3° of the set temperature by use of cooling and heating units controlled by a bimetallic sensing device.

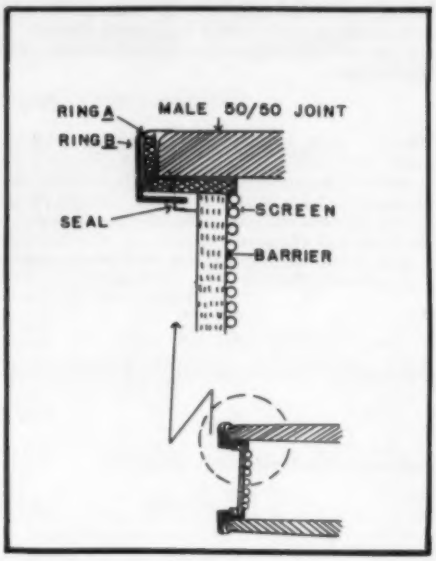


Fig. 3.—Diagram of the barrier showing the method of sealing.

### Procedure

**Preparation of the films.**—*Films from Cellulose Esters.*—Cellulose ester films were cast from their solutions. These were prepared by dissolving the polymer flakes by stirring and shaking overnight in solvents suggested by Antoinides and DeKay (9), to make a 10 to 20% solution by weight.

*Plastic Films.*—These films were obtained directly from the manufacturers and were used as such.

*Glyceryl Stearate Films.*—Films of chromatographically purified (10) glyceryl stearates were made by casting on mercury.

**Measurement of Film Thickness.**—The thickness of the plastic and cellulose ester films was measured with a micrometer caliper. Twenty-five readings of the thickness spread out over the surface of the film were taken and the average of these was used as the effective thickness.

To prevent defacing the wax films, a slightly different procedure was followed in obtaining their thickness. To insure uniformity of the cast, film thicknesses of regions around the center of the film were determined with the micrometer caliper. A disk was then cut off from the center of the film and the thickness of the rim of the disk was measured with an optical micrometer.

**Procedure for Measurement of Permeability.**—To make a determination, stopcocks B, C, D, and F (Fig. 2) were fully open initially and stopcock E was used to control the initial rate of evacuation to prevent rupture of the film. After deaerating the water in the flask by alternately freezing and allowing to come to room temperature, stopcock B was

shut off. The system was then evacuated to about $1 \times 10^{-3}$ mm. of mercury pressure and the evacuation was carried on for about twenty-four hours to remove traces of water from the film. After this period stopcocks C, D, and E were shut off.

Before actual permeation measurements were begun, readings were taken for approximately an hour to make sure that no vapor was being given off from the films which would give erroneous results, and that the apparatus was not subject to leaks.

At zero time stopcock B was opened allowing the water vapor from the reservoir to come into contact with one face of the film. The change in pressure on the other side of the barrier was followed with the McLeod gauge.

## RESULTS AND DISCUSSION

### Permeation Through Various Materials

**Permeation Through Cellulose Esters.**—Results obtained in studying rates of permeation through films formed from cellulose acetate hydrogen phthalate, cellulose triacetate, cellulose acetate propionate, cellulose acetate butyrate, and cellulose acetate stearate are shown in Figs. 4, 5, and 6. The permeability and diffusion coefficients calculated from these plots as described above are given in Table I. The thickness of the films and some such values reported in the literature are also given for comparative purposes.

From the data it is evident that as the molecular weight of the ester groups in the cellulose or, equivalently, the proportion of the aliphatic content of the film increases, the permeability and the apparent diffusion coefficient calculated from lag time decrease markedly. The decrease in the former coefficient with increasing aliphatic content of the film can be probably ascribed to the decrease in the polarity of the film. The dependence of the rate of permeation on the polarity of the film arises from the solubility coefficient $S$ of the permeability equation. In a more polar film the affinity of the water molecules for the barrier phase is greater, hence the solubility coefficient will be increased. This will increase the rate of permeation to the same extent. As the polarity of the film is decreased by the addition of large aliphatic substituents, the rate of permeation will, conversely, decrease.

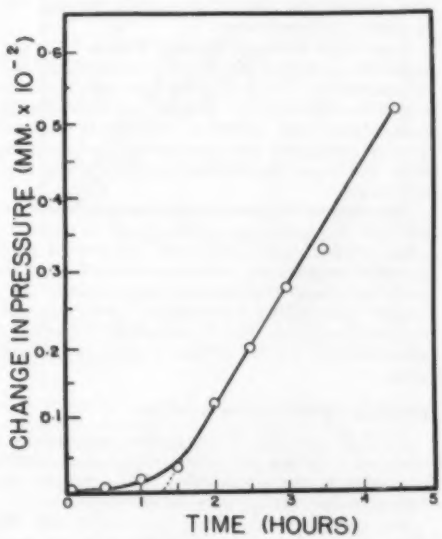


Fig. 4.—Plot showing permeation through cellulose acetate hydrogen phthalate (0.1322 cm. thick).

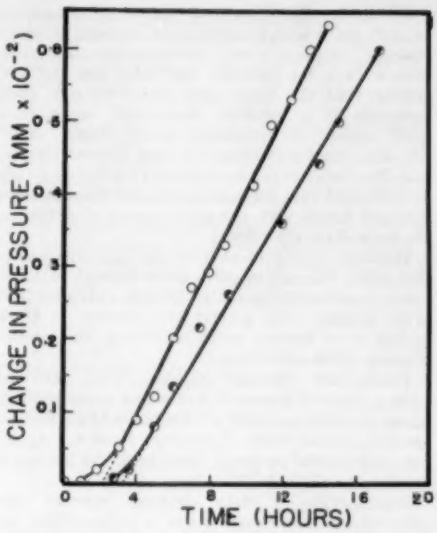


Fig. 5.—Plot showing permeation through ○—cellulose triacetate (0.1521 cm. thick) and ◐—cellulose acetate propionate (0.1625 cm. thick).

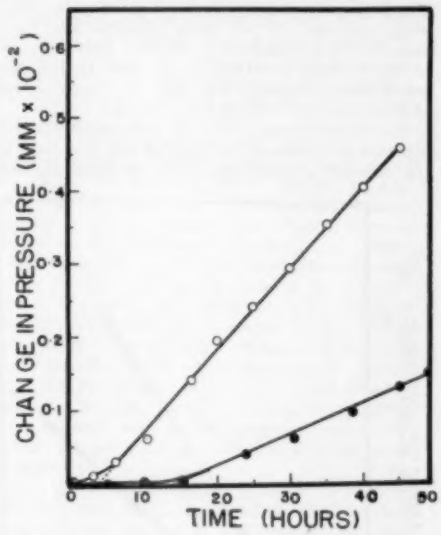


Fig. 6.—Plot showing permeation through ○—cellulose acetate butyrate (0.1666 cm. thick) and ●—cellulose acetate stearate (0.1631 cm. thick).

Much has been written in the literature as regards the actual mechanism of passage of water through cellulose and cellulose-like materials. Waack (12), for instance, has ruled out the possibility that the water permeation through these materials is a capillary movement, since their void volume is extremely small. Doty, *et al.* (8), suggests the existence of very narrow crevices and slits between the crystallites of cellulose esters. It is thought that the water permeates through these slits and bonds with the polar groups available on the walls of the crystallites.

However, as is evident from the data obtained in this study, the rate of permeation through cellulose esters is primarily governed by the availability of polar groups. The greater the number of these groups other factors notwithstanding, the greater the rate of passage of water.

**Permeation Through Plastics.**—Data obtained from a study of permeation of water vapor through Saran A, Koroseal, and polytrifluorochloroethylene are presented in Table II and Figs. 7 and 8. Again, for comparative purposes, data from the literature are included in Table II.

Examination of data obtained reveals that polytrifluorochloroethylene has a permeability coefficient one-tenth that of Saran A and approximately one-hundredth that of Koroseal. The diffusion coefficient of polytrifluorochloroethylene is also smaller than that of Saran A and Koroseal. This behavior of polytrifluorochloroethylene is of some interest. In all these plastics the basic structure is a hydrocarbon skeleton and there are no hydrophilic substituents or side chains, yet polytrifluorochloroethylene has a much lower permeability than the other two plastics.

The lower permeability of polytrifluorochloroethylene as compared to Saran A and Koroseal, in the absence of polar groups in all three plastics,

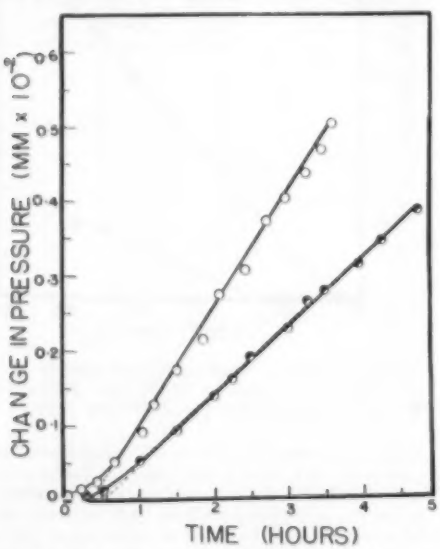


Fig. 7.—Plot showing permeation through ○—Koroseal (0.0031 cm. thick) and ◐—Saran A (0.0048 cm. thick).

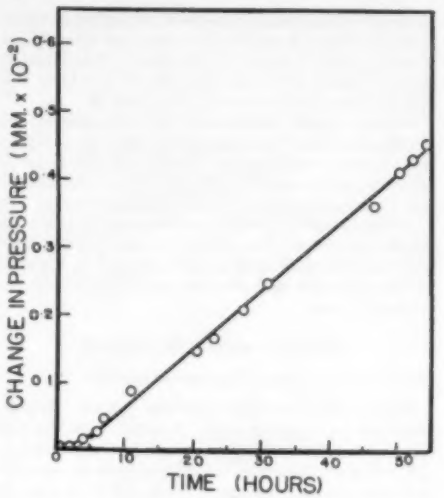


Fig. 8.—Plot showing permeation through polytrifluorochloroethylene.

can be attributed to more even packing of trifluorochloroethylene molecules or their extremely nonpolar character. In a polymer like polyvinylidene chloride, chlorine atoms are opposed by hydrogen atoms which differ greatly in size, making the packing of the molecules uneven. Frey, and others (13), have described polytr fluorochloroethylene as polyvinylidene chloride in which all the hydrogen atoms are replaced by the larger fluorine molecules thus making the outward contour of the molecule more uniform. The uniformity of the molecules leads to a better packing of the material preventing any cracks, etc., through which the water may flow, consequently the rate of permeation through this material is extremely slow.

**Permeation Through Glyceryl Waxes.**—The permeability coefficient for glyceryl monostearate was determined as $12.9 \times 10^{-9}$ and the diffusion coefficient was $194 \times 10^{-9}$. Attempts to determine the permeability and diffusion coefficients of pure glyceryl distearate and tristearate failed, as these films developed longitudinal cracks during their evaluation.

Comparison of the permeability coefficients of glyceryl monosterarate with those of cellulose esters studied again emphasizes the role of polar groups in permeation. Cellulose esters being more polar in general than glyceryl monostearate have larger permeability coefficients. For example, cellulose triacetate has a permeability coefficient approximately ten times that of glyceryl monostearate.

### Dependence on Relative Humidity

Table III and Fig. 9 present data obtained on a study of variation of vapor pressure and relative humidity on the permeability and apparent diffusion coefficients of glyceryl monostearate.

For systems which follow Henry's law and also show independence of diffusion coefficient with respect to concentrations, experimentally deter-

TABLE I.—PERMEABILITY AND DIFFUSION COEFFICIENTS OF SOME CELLULOSE ESTERS

| Material | Thickness, cm. | Observed $D^a \times 10^6$ | Observed $P^b \times 10^6$ | Literature Values $D^a \times 10^6$ | Literature Values $P^b \times 10^6$ |
|---|---|---|---|---|---|
| Cellulose acetate hydrogen phthalate | 0.1322 | 62.0 | 325 | ... | ... |
| Cellulose triacetate | 0.1521 | 53.6 | 133 | 58.2 | ... |
| Cellulose acetate propionate | 0.1625 | 36.7 | 108 | ... | 102.0(11) |
| Cellulose acetate butyrate | 0.1666 | 25.7 | 42 | ... | 52.0(11) |
| Cellulose acetate stearate | 0.1631 | 8.5 | 10.5 | ... | ... |

[a] Diffusion coefficient in cm.$^2$ sec.$^{-1}$. [b] Permeability coefficient in cc./sec./cm.$^2$/cm.Hg/cm. thick.

TABLE II.—PERMEABILITY AND DIFFUSION OF COEFFICIENTS OF PLASTICS

| Material | Thickness, cm. | Observed Values $D^a \times 10^6$ | Observed Values $P^b \times 10^6$ | Literature Values $D^a \times 10^6$ | Literature Values $P^b \times 10^6$ |
|---|---|---|---|---|---|
| Saran A, polyvinylidene chloride | 0.0048 | 0.21 | 0.05 | 0.125(8) | 0.07(8) |
| Koroseal, polyvinyl chloride | 0.0031 | 0.12 | 7.7 | ... | 5.6(8) |
| Polytrifluorochloroethylene | 0.0048 | 0.02 | 0.006 | ... | ... |

[a] Diffusion coefficient in cm.$^2$ sec.$^{-1}$. [b] Permeability coefficient in cc./sec./cm.$^2$/cm. Hg/cm. thick.

TABLE III.—EFFECT OF CHANGE OF RELATIVE HUMIDITY ON GLYCERYL MONOSTEARATE

| Humidity[a], % | $P^b \times 10^8$ | $D^c \times 10^6$ | $S^d \times 10^2$ | Thickness, cm. |
|---|---|---|---|---|
| 100 | 12.9 | 194 | 6.64 | 0.1026 |
| 80 | 7.4 | 206 | 3.59 | 0.1021 |
| 52 | 5.2 | 165 | 3.15 | 0.1036 |
| 31 | 4.4 | 145 | 3.03 | 0.1073 |

[a] At 24.5°. [b] Permeability coefficient in cc./sec./cm.$^2$/cm. Hg/cm. thick. [c] Diffusion coefficient in cm.$^2$ sec.$^{-1}$. [d] Solubility coefficient in cc. of gas/cc. of film material.

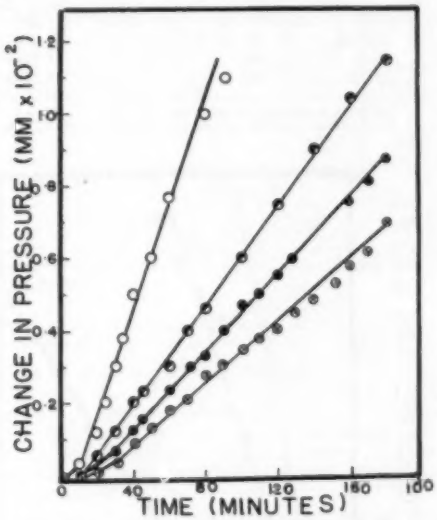


Fig. 9.—Plot showing effect of change of relative humidity on permeation through glyceryl monostearate. ○—100% humidity (0.1026 cm. thick), ◐—80% humidity (0.1021 cm. thick), ●—52% humidity (0.1036 cm. thick), ⊗—31% humidity (0.1073 cm. thick).

mined values of $P$ and $D$ should be independent of the partial pressure of the penetrating vapor. This relationship apparently does not hold for glyceryl monostearate-water systems as is evident from the data. The rate of transfer of water vapor through this material appears to decrease much faster than the pressure of water vapor on this high pressure side.

This behavior unfortunately casts some doubt as to the absolute validity of the use of the lag time method in determining diffusion coefficients in these systems. It can be shown readily that if the permeability coefficients exhibit concentration dependency, then the diffusion coefficients determined in this fashion are only apparent. One must be concerned, for example, with the obvious tendency for materials of this type to bind water molecules which are not kinetically equally available for the diffusion process. This behavior alone will always give much too low a diffusion coefficient when calculated from lag time. Mathematical relationships in a similar system have already been developed but will be reported elsewhere.

The variation of the diffusion constants with change in vapor pressure in glyceryl monostearate appears to parallel the findings of Lowry and Kohman (14) in their work of solution of water in rubber. They state, "The solubility of water in rubber at vapor pressure below approximately 16 mm. at 25° is directly proportional to the first power of the pressure. At these pressures, then, the solubility obeys Henry's law, but at pressures higher than 16 mm., the solubility is much greater than the law would predict."

If a similar situation exists in the glyceryl monostearate system, Fick's linear diffusion law would be obeyed in the regions of low relative humidity. At high relative humidities the high concentration of water on the wet side of the membrane would result in lower apparent diffusion coefficient than would be expected.

Plots of permeability and diffusion coefficients *vs.* per cent humidity shown in Fig. 10 serve to emphasize the arguments presented above. As is evident from the figure, the apparent diffusion coefficient apparently reaches a maximum at 80% humidity and then decreases, instead of increasing as expected from theoretical considerations. The permeability coefficient on the other hand shows a greater rate of increase on passing from 80% hu-

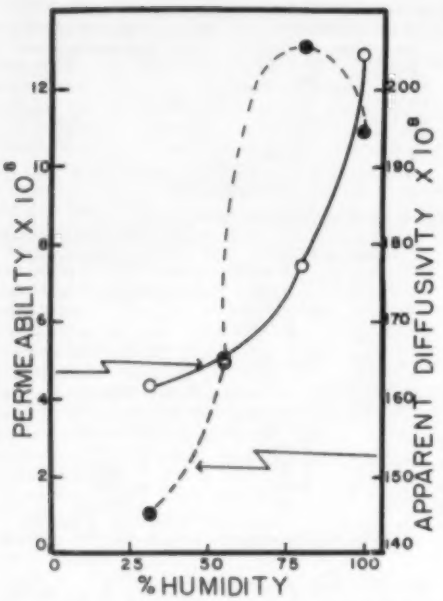


Fig. 10.—Plot showing variation of permeability and apparent diffusivity of glyceryl monostearate with change in relative humidity.

midity to 100%. The apparent reduction at 100% relative humidity in the apparent diffusion coefficient is probably due to a large increase in the number of water molecules bound by the wax phase.

Although a similar study on the cellulose esters was not carried out, a like situation probably exists. Both systems tend to sorb and bind water molecules significantly. In such cases diffusion coefficients estimated from lag time can only be considered apparent. It is presumed that if higher humidity was permitted to exist on the low pressure side of the barriers, the observed effective permeability would have been considerably higher.

### Temperature Dependence of Permeation

Barrer (1) has conclusively shown that the permeation of gases and vapors through a film requires an energy of activation to make the molecules enter the pores and move along the path to the other surface. This he has expressed in an Arrhenius type of equation

$$P = P_0 e^{-\frac{E_p}{RT}} \qquad \text{(Eq. 11)}$$

where $P$ is the permeability constant. $P_0$ is a factor, independent of temperature, which is proportional to the number of molecules that are available to enter the pore structure and also to the probability that a molecule having a sufficient energy will actually enter this pore. $P_0$ may also be defined as the permeability constant at absolute zero. $E_p$ is the energy of activation for permeation. $R$ is gas constant and $T$ absolute temperature.

The equation for temperature dependence of diffusion is also given by an Arrhenius type of equation and can be written as

$$D = D_0 e^{-\frac{E_D}{RT}} \qquad \text{(Eq. 12)}$$

where $D$ is the diffusion coefficient, $D_0$ is a constant independent of temperature or the diffusion coefficient at absolute zero, and $E_D$ is the energy of activation for diffusion.

If $P$ behaves as Eq. 11 and $D$ behaves as Eq. 12, and since $P = -DS$, it is necessary that the solubility coefficient $S$ exhibit the same functional variation with temperature. Hence it would require that

$$S = S_0 e^{\frac{H}{RT}} \qquad \text{(Eq. 13)}$$

where $S_0$ is a constant and $H$ is the heat of solution of water vapor in the membrane.

The variation of the diffusion and permeability coefficients of glyceryl monostearate with temperature is shown in Table IV and Fig. 11.

The activation energy for permeation was

TABLE IV.—EFFECT OF TEMPERATURE VARIATION ON THE PERMEABILITY AND APPARENT DIFFUSIVITY THROUGH GLYCERYL MONOSTEARATE

| Temperature, °C. | $D^a \times 10^8$ | $P^b \times 10^8$ | Thickness, cm. |
|---|---|---|---|
| 16.9 | 91.5 | 8.9 | 0.1047 |
| 24.3 | 194 | 12.9 | 0.1006 |
| 29.5 | 333 | 16.2 | 0.1039 |

[a] Diffusion coefficient in cm.² sec.⁻¹.
[b] Permeability coefficient in cc./sec./cm.²/cm. Hg/cm. thick.

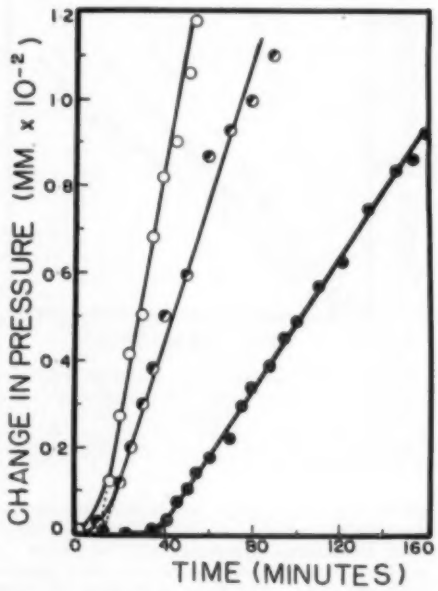


Fig. 11.—Plot showing temperature dependence of permeation through glyceryl monostearate.
○—G. monostearate (0.1047 cm. thick) at 16.9°.
◐—G. monostearate (0.1006 cm. thick) at 24.3°.
●—G. monostearate (0.1039 cm. thick) at 29.5°.

obtained from the slope of a plot of log of permeation *vs.* the reciprocal of the absolute temperature shown in Fig. 12.

The activation energy of permeation was found to be 7,700 cal./mole and the apparent activation energy for diffusion was 16,400 cal./mole. By difference, the heat of solution was calculated and the value obtained was 8,700 calories, a very reasonable value.

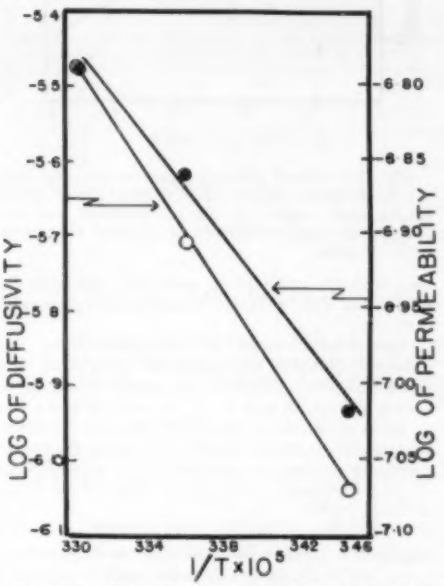


Fig. 12.—Plot of log of diffusivity and log of permeability *vs.* the reciprocal of absolute temperature from which the activation energies were calculated.

### Permeation Through Mixtures of Glyceryl Stearate and White Wax

Addition of a relatively impermeable substance like white wax to the glyceryl stearates tends to enhance their water resistance. This is evident from Tables V, VI, and VII, and also from Figs. 13, 14, and 15, where experimental results obtained on the several stearates admixed with white wax are presented.

Decrease of the permeability coefficient of glyceryl stearates on progressive increase of concentration of white wax can again be ascribed to the decrease in the effective solubility coefficient of the permeability equation. The solubility coefficient would decrease since the addition of white wax would eliminate some of the active centers where the water molecules could bond, i. e., the free hydroxyl groups in the glycerides.

Since glyceryl monostearate has a higher number of these active centers the rate of the decrease of the permeability coefficient would be much greater than glyceryl di- or tristearate. This is borne out from a comparison of data in Tables V, VI, and VII.

It is apparent from Table VI that the apparent diffusion coefficient of glyceryl monostearate remains essentially constant with increase of concentration of white wax up to 30%, after which it drops markedly. This may be due to possible formation of a two-phase system by the two components corresponding to that concentration of white

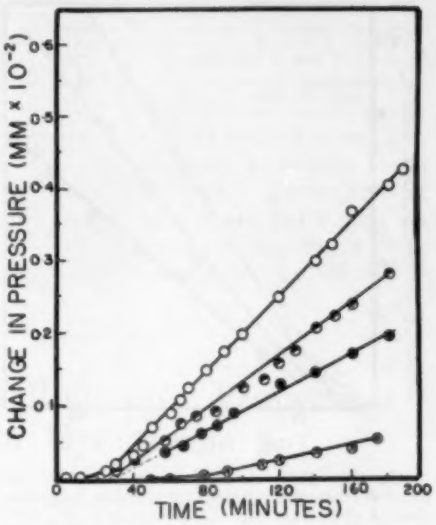


Fig. 13.—Plot showing permeation through glyceryl monostearate admixed with different concentrations of white wax. ○—G. monostearate + 10% white wax (0.2172 cm.). ◐—G. monostearate + 20% white wax (0.2021 cm.). ●—G. monostearate + 30% white wax (0.2094 cm.). ⊗—G. monostearate + 40% white wax (0.2136 cm.).

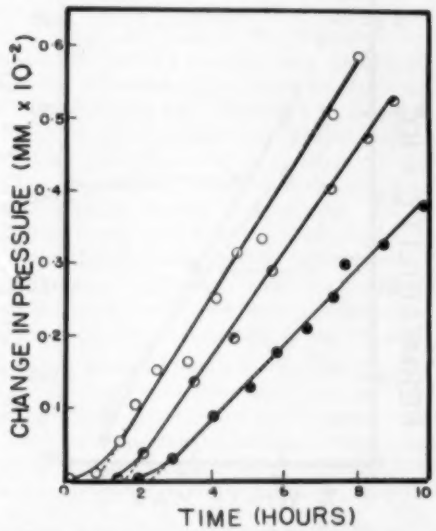


Fig. 14.—Plot showing permeation through glyceryl distearin admixed with various concentrations of white wax. ○—G. distearate + 10% white wax (0.2031 cm.). ◐—G. distearate + 20% white wax (0.2081 cm.). ●—G. distearate + 30% white wax (0.2060 cm.).

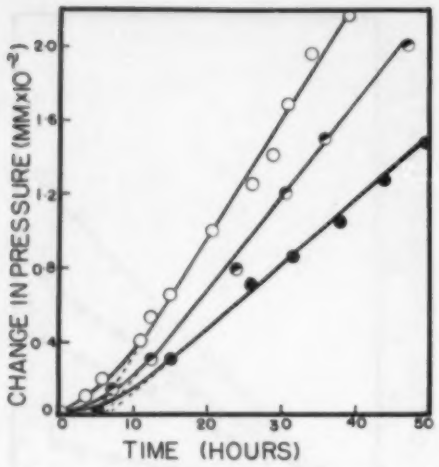


Fig. 15.—Plot showing permeation through glyceryl tristearate admixed with various concentrations of white wax. ○—G. tristearate + 20% white wax (0.1089 cm.). ◐—G. tristearate + 30% white wax (0.1125 cm.). ●—G. tristearate + 50% white wax (0.0993 cm.).

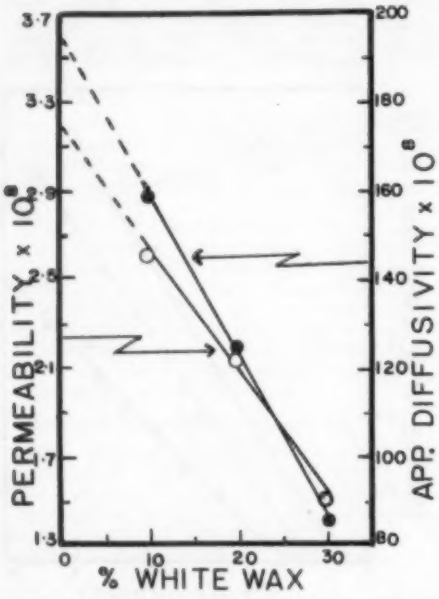


Fig. 16.—Plots showing extrapolation to zero per cent white wax concentration from which approximate values of the permeability and apparent diffusion coefficients of glyceryl distearate were obtained.

wax necessary to form a more or less continuous phase.

Of some interest is the behavior of the diffusion coefficient of glyceryl di- and tristearate on addition of increasing concentration of white wax. Tables VI and VII indicate that the diffusion coefficient

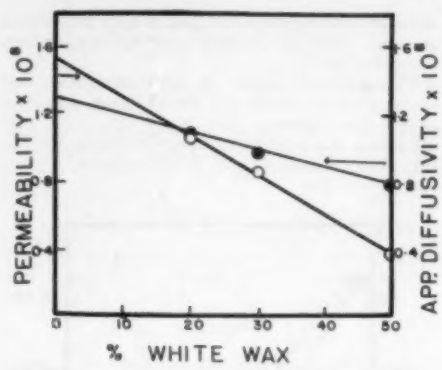


Fig. 17.—Plots showing extrapolation to zero per cent white wax concentration from which approximate values of the permeability and apparent diffusion coefficients of glyceryl tristearate were obtained.

falls with the addition of white wax although the rate of fall with tristearin is much greater than with distearin.

Approximate values of the permeability and apparent diffusion coefficients of glyceryl di- and tristearate were obtained by extrapolating plots, shown in Figs. 16 and 17, of per cent of white wax *vs.* these constants to zero white wax concentration. As mentioned earlier, direct determination of these constants was not possible due to the brittleness of di- and tristearin. The values obtained are given in Table VIII.

TABLE V.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL MONOSTEARATE

| White Wax, % | Thickness, cm. | $D^a \times 10^8$ | $P^b \times 10^8$ |
|---|---|---|---|
| 10 | 0.2172 | 403 | 5.5 |
| 20 | 0.2021 | 404 | 3.5 |
| 30 | 0.2094 | 405 | 2.3 |
| 40 | 0.2136 | 171 | 1.0 |

[a] Diffusion coefficient in cm.² sec.⁻¹.
[b] Permeability coefficient in cc./sec./cm.²/cm. Hg/cm. thick.

TABLE VI.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL DISTEARATE

| White Wax, % | Thickness, cm. | $D^a \times 10^8$ | $P^b \times 10^8$ |
|---|---|---|---|
| 10 | 0.2031 | 159 | 2.6 |
| 20 | 0.2081 | 125 | 2.1 |
| 30 | 0.2060 | 86.4 | 1.5 |

[a] Diffusion coefficient in cm.² sec.⁻¹.
[b] Permeability coefficient in cc./sec./cm.²/cm. Hg/cm. thick.

TABLE VII.—EFFECT OF ADDITION OF WHITE WAX TO GLYCERYL TRISTEARIN

| White Wax, % | Thickness, cm. | $D^a \times 10^8$ | $P^b \times 10^8$ |
|---|---|---|---|
| 20 | 0.1089 | 1 | 1 |
| 30 | 0.1125 | 0.97 | 0.85 |
| 50 | 0.0993 | 0.78 | 0.37 |

[a] Diffusion coefficient in cm.² sec.⁻¹.
[b] Permeability coefficient in cc./sec./cm.²/cm. Hg/cm. thick.

TABLE VIII.—PERMEABILITY AND DIFFUSION COEFFICIENTS OF GLYCERYL DI- AND TRISTEARATE

| | $D^a \times 10^8$ | $P^b \times 10^8$ |
|---|---|---|
| G. distearate | 204 | 3.21 |
| G. tristearate | 13 | 1.54 |

[a] Diffusion coefficient in cm.$^2$ sec.$^{-1}$.
[b] Permeability coefficient in cc./sec./cm.$^2$/cm. Hg/cm. thick.

## REFERENCES

(1) Barrer, R. M., "Diffusion In and Through Solids," Cambridge University Press, Cambridge, England, 1951.
(2) Lueck, L. M., University of Wisconsin, Ph.D. Thesis, 1954.
(3) Jost, W., "Diffusion in Solids, Liquids, Gases," Academic Press Inc., New York, N. Y., 1952.
(4) Glasstone, S., "Textbook of Physical Chemistry," 2nd ed., D. Van Nostrand, Inc., New York, N. Y., 1946.
(5) Taylor, R. L., Herrmann, D. B., and Kemp. A. R., *Ind. Eng. Chem.*, **28**, 1255(1936).
(6) Barrer, R. M., *Trans. Faraday Soc.*, **35**, 628(1939).
(7) Daynes, H., *Proc. Royal Soc.*, **97A**, 286(1920).
(8) Doty, P. M., Aiken, W. H., and Mark, H., *Ind. Eng. Chem., Anal. Ed.*, **16**, 686(1944).
(9) Antoinides, H. J., and DeKay, H. G., *Drug Standards*, **21**, 205(1953).
(10) Papariello, G., Higuchi, T., Martin, J. B., and Kuceski, V. P., in press.
(11) Deeg, G., Jr., and Frosch, C. J., *Modern Plastics*, **22**, 198(1944).
(12) Wanck, R., Alex, N. H., and Frisch, H. L., *Ind. Eng. Chem.*, **47**, 2524(1955).
(13) Frey, S. E., Gibson, J. D., and Lafferty, R. H., Jr., *ibid.*, **42**, 2314(1950).
(14) Lowry, H. H., and Kohman, G. J., *J. Phys. Chem.*, **31**, 23(1927).

# Some Physical and Crystallographic Properties of the Ouabain Hydrates*

By JAGDISH TRIVEDI, JOHN W. SHELL†, and JOHN A. BILES

**Different hydrates of ouabain have been isolated. The physical and crystallographic properties of the hydrates as well as the X-ray powder diagrams are reported and discussed. The solubility of ouabain is reported for two different water-alkanol systems.**

THE EXAMINATION of the available data on the isolation of and the stability of possible hydrates of ouabain indicates the data to be confusing. There is also confusion existing with respect to the optical crystallographic data of the hydrates. Thus, the purpose of this communication is to clarify the methods for isolating the different hydrates, to report the optical crystallographic properties of the hydrates, and to record the X-ray powder diffraction data.

The crystallographic properties of ouabain have been reported by Keenan and co-workers (1, 2), Shell and Witt (3), and Kofler (4). Keenan and co-workers reported the isolation of three different hydrates of ouabain. Ouabain with 19.2 per cent water was recrystallized from hot water; and ouabain containing 11.9 per cent water was recrystallized from 95 per cent ethanol. The refractive indexes of these hydrates were recorded (1). Shell and Witt determined the optical crystallographic data for ouabain recrystallized from ethanol.

Kofler reported the isolation of the decahydrate and octahydrate of ouabain when recrystallized from a cooled aqueous solution. He stated that the trihydrate was isolated from hot water or when acetone was added to the octahydrate and allowed to stand for several hours. Siewart (5) reported the isolation of the trihydrate of ouabain by dissolving 0.5 Gm. of ouabain in 17 cc. of dioxan and 0.3 cc. of water and allowing to stand at room temperature for about two days. Kofler further reported the isolation of the dihydrate from acetone, alcohol, and aqueous sodium chloride (1 per cent) solution. The addition of sodium chloride to an alcoholic solution was a simple means of isolating the dihydrate. Kofler stated that the ouabain dihydrate was unstable in an aqueous solution but stable in the dry state.

It was concluded from a study of the literature that the octahydrate, the trihydrate, and the hydrate from alcohol were stable, whereas the decahydrate was unstable and the dihydrate was stable under certain given conditions. The melting points of all the hydrates excepting the trihydrate were reported to be 180–185°. The trihydrate melted at 240–245°.

The solubility of ouabain in water was reported by Arnaud (6) and Richaud (7). Additional solubilities are listed in the U. S. P. XV (8) and the Merck Index (9). Several workers have reported ouabain to be stable (10–13). Berry reported that ouabain was stable over a wide pH range (14).

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Abstracted in part from a dissertation by Jagdish Trivedi, submitted to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.
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## EXPERIMENTAL

**Materials.**—Ouabain, U. S. P.; ethanol, U. S. P.; and isopropanol, N. F., were used in preparing the alkanol-water solutions for the solubility determinations. Ethanol, U. S. P., methanol, C. P., and dioxane, C. P., were used as crystallizing solvents. Sodium chloride, C. P., was used in crystallization of ouabain. Karl Fischer reagent was prepared according to U. S. P. XV directions.

**Apparatus.**—A Karl Fischer apparatus similar to that described in U. S. P. XV was used to determine the water content of some ouabain hydrates. The water content of the different phases was also determined using an Abderhalden apparatus, charged with phosphorus pentoxide. The boiling solvent was bromobenzene, b. p. 150–155°. The solubility of the ouabain in the different alkanol solvents was determined using a rotating wheel apparatus which held 20-cc. vials. The wheel was rotated by a motor and was submerged in a constant temperature bath. Rotation was continuous for eight hours.

**Crystallization of the Ouabain Hydrates.**—The ouabain was placed in the solvent for crystallization. An excess of ouabain was used, and the solvent was heated above the crystallization temperature. The solution was allowed to cool to the temperature desired for crystallization following filtration. After a sufficient amount of the ouabain had crystallized in the container, the heterogenous system was closed to prevent further evaporation of the solvent. The system was allowed to equilibrate for twenty-four hours. In this manner, ouabain was recrystallized at 0° and at intervals of 10° from 20 through 90°. The recrystallization at elevated temperatures was done in an oven.

Ouabain was recrystallized from ethanol by saturation of a heated solution and allowed to cool. Ouabain was allowed to crystallize from 6% water in dioxane by heating the solution, filtering, and allowing to cool. Ouabain was crystallized from 97% methanol and also from a 50% ethanol solution containing 1% sodium chloride.

**Determination of Water Content in the Ouabain Hydrates.**—In the initial determination of per cent water in the hydrates, the Karl Fischer method of water determination was used. However, the method required the use of rather large amounts of the ouabain. Therefore, the loss of weight of heating the hydrates in the Abderhalden apparatus was checked against the Karl Fischer method. The precision was close enough to justify the use of determination of water content by loss in weight. The recovery of ouabain was greater using the loss in weight method.

**Optical Crystallographic Properties.**—The optical crystallographic data were determined by means of a petrographic microscope. A Scopelite (Clay Adam Co., N. Y.) containing a 25-watt bulb and blue filter was used as a light source. The method of determination of the data was similar to that described by Biles, Witt, and Poe (15).

**Photomicrographs.**—The crystals were mounted on the petrographic microscope. A Bausch and Lomb photomicrographic camera model L was used in obtaining the photomicrographs.

**Melting Points.**—The melting points were determined using a Kofler melting point block mounted on a microscope stage. The crystals were observed under 80 × magnification.

**X-ray Powder Diagrams.**—The diameter of the camera was such that the mm. distance between arcs on the film was equivalent to a degree. Copper radiation, filtered by nickel, was used. A sliding scale with an attached vernier was used to measure the distance between a given pair of arcs.

**Solubility Determinations and Assay.**—Duplicate samples were used in determining the solubility of ouabain in ethanol-water and isopropanol-water solutions. The percentages w/w of the solutions were determined by specific gravity determinations using a Westphal balance, and refractive index determinations using an Abbe refractometer. The values were compared with the values reported in the International Critical Tables (16).

An excess of ouabain was placed in a 20-cc. vial. The solvent was added and the vial stoppered with a rubber seal. Eight vials were placed in a plastic wheel. The wheel was rotated in a constant temperature bath for eight hours. The vials were removed and the solution filtered. The filtrate was rapidly collected and the solution diluted and assayed using the U. S. P. XV procedure for assay. A Beckman model B spectrophotometer was used for the colorimetric method of analysis.

## RESULTS AND DISCUSSION

**Ouabain Hydrates.**—Six different hydrates of ouabain were isolated. The melting point, water content, and the temperature of isolation are recorded in Table I. The temperature-water composition phase diagram for the hydrates isolated from water is illustrated in Fig. 1.

The nonahydrate was isolated by crystallization at temperatures of 0–15°. The nonahydrate was not observed when ouabain was crystallized from

TABLE I.—WATER CONTENT, M. P., AND TEMPERATURE OF ISOLATION OF THE OUABAIN HYDRATES

| Hydrate | Water Calculated, % | Water Experimental,[a] % | M. p., °C. | Temperature of Isolation, °C. |
|---|---|---|---|---|
| Ouabain·$9H_2O$ | 21.71 | 21.93 | 190–195 | 0–15 |
| Ouabain·$8H_2O$ | 19.78 | 20.13 | 190–195 | 15–28 |
| Ouabain·$4^1/_2H_2O$ | 12.18 | 12.10 | 185–187 | 20–30[c] |
| | | 11.84[b] | | |
| Ouabain·$4H_2O$ | 10.98 | 10.88 | 190–195 | 20–30[d] |
| Ouabain·$3H_2O$ | 8.46 | 8.43 | 240–247 | 20–30[e] |
| Ouabain·$2H_2O$ | 5.80 | 6.02 | 185–195 | 28–90 |
| Ouabain | ... | ... | 190–195 | 150[f] |

[a] Determined by loss of weight of water in Abderhalden apparatus at 150°. Average of duplicate determinations. [b] Karl Fischer method of determination. [c] From 95% ethanol. [d] From 97% methanol. [e] From dioxane containing 6% water. [f] Obtained by heating ouabain recrystallized from ethanol in Abderhalden apparatus at 150°.

water at room temperature. This phase evidently corresponded to the decahydrate reported by Kofler (4). Attempts were not successful in isolating the decahydrate. The octahydrate was isolated at room temperature. The octahydrate and the dihydrate existed in equilibrium at 28° in aqueous solution. This was repeated several times since Schwartze (1) reported isolating the trihydrate from hot water and Kofler (4) stated that the trihydrate was the most stable hydrate in saturated aqueous solution at a temperature of about 70°. When the dihydrate was isolated from the aqueous solution, dried between filter paper, and the material subjected to drying in the Abderhalden, there was a water loss of 8.12%. However, the X-ray powder patterns for the material dried between filter paper and the material subjected to drying at 130° were the same. Therefore, it might be possible that Schwartze and Kofler considered the material isolated at 70° to be the trihydrate, whereas the present investigation indicates that the dihydrate contains excess water which is not part of the repeat lattice. The dihydrate was isolated from aqueous solutions at temperatures of 28° up to 90°. Above 90° isotropic resin-like material was isolated.

Kofler's work was verified with respect to isolation of ouabain from ethanol. Similar results were reported by Schwartze. Water composition studies indicated that the crystal contained an equivalent of 4.5 moles of water per mole of ouabain. When ouabain was crystallized from 97% methanol, water loss indicated that the crystal contained an equivalent of 4 moles of water per mole of ouabain. The optical crystallographic properties were very similar in all respects except for the principal refractive indexes. The X-ray patterns for these powders from ethanol and methanol were the same. It was concluded, therefore, that the one-half mole of water in the crystals from ethanol was not part of the repeat lattice. This extra water may be held by entrapment by hydrogen bonding. Therefore, it may be possible that optical crystallographic properties can detect solvated phases to which X-ray powder patterns are insensitive.

**Optical Crystallographic Data.**—The data are recorded in Table II. The properties of the hydrate from ethanol corresponded to those values recorded by Keenan. The properties of the hydrate from 97% methanol were similar to those recorded by Shell and Witt. The octahydrate was determined to be monoclinic with the $\alpha$- and $\beta$-refractive indexes corresponding to the values reported by Keenan. However, Keenan reported the octahydrate to be tetragonal; the octahydrate appears to be pseudotetragonal. Kofler reported the refractive indexes of the trihydrate. His values indicated the crystal to be optically negative. On orientation of the bisectrix, the trihydrate was determined to be positive. The only point of difference was the reported value for the alpha index. The anhydrous form was determined to be isotropic. The refractive index corresponded to that reported by Keenan—namely 1.565. When the dihydrate was subjected to drying in the Abderhalden at 150°, the optical crystallographic data were redetermined. Some data indicated the crystals differed from the dihydrate before drying. X-ray powder patterns of the two crystals were the same.

The crystal habits and systems are very similar for most of the hydrates. These data justify the statement made by Kofler that on heating, the optical properties do not change. Futhermore, this explains the failure to notice changes in crystalline phases on the Kofler block and the similarity

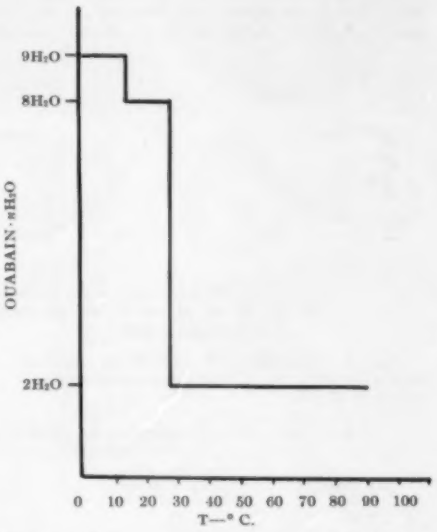



Fig. 1.—Temperature-water composition phase diagram for ouabain hydrates.

TABLE II.—THE OPTICAL CRYSTALLOGRAPHIC PROPERTIES OF THE HYDRATES OF OUABAIN

| Hydrate | Crystal Habit | Crystal System | Optic Orientation[c] | Optic Sign | Axial Angle[a] | Dispersion | Common Orientation | Refractive Indexes $\alpha$ | $\beta$ | $\gamma$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Ouabain·9H₂O | Tabular | Orthorhombic | XX ‖ a<br>YY ‖ b | − | 59° | None | Bxa, off Centered | 1.502 | 1.521 | 1.532 |
| Ouabain·8H₂O | Tabular | Monoclinic | XX ‖ b | − | 38° | Crossed (Bxo) | Bxa, off Centered | 1.521 | 1.525 | 1.528 |
| | | | YY ∧ c 33° | | | | | | | |
| Ouabain·4½H₂O | Columnar | Orthorhombic | XX ‖ a, YY ‖ b | + | 55° | None | Bxo | 1.531 | 1.542 | 1.584 |
| Ouabain·4H₂O | Tabular | Orthorhombic | XX ‖ a, YY ‖ b | + | 33° | None | Bxo | 1.533 | 1.536 | 1.570 |
| Ouabain·3H₂O | Acicular | Orthorhombic | XX ‖ a, YY ‖ b | + | 74° | v > r, axial | Bxo, off Centered | 1.571 | 1.582 | 1.602 |
| Ouabain·2H₂O | Columnar | Orthorhombic | XX ‖ a, YY ‖ b | + | 41° | v > r, axial | Bxa, off Centered | 1.557 | 1.560 | 1.582 |
| Ouabain·2H₂O[b] | Columnar | Orthorhombic | XX ‖ a, YY ‖ b | + | 43° | v > r, axial | Bxo | 1.550 | 1.553 | 1.573 |

[a] 2V, calculated. [b] The dihydrate dried at 150° for four hours. [c] Assigned according to crystal habit.

in melting points of the different phases. The formation of the anhydrous phase from the trihydrate and dihydrate is least practical using the Abderhalden apparatus and a boiling solvent of 150°.

**X-ray Powder Patterns.**—The interplanar spacings in Å. were calculated and recorded in Table III. The three strongest lines observed on the films are ranked in order of intensity by 1, 2, and 3. Examination of ouabain·$4\frac{1}{2}H_2O$ (from ethanol) and ouabain·$4H_2O$ (from methanol) powder *d*-distances indicate similar lattices. The same is true for the dihydrate and the dihydrate dried in the Abderhalden. It has been suggested that the X-ray powder patterns are insensitive to some solvated phases. This suggests water entrapment by hydrogen bonding. It is thought that proton resonance spectra will clarify the differences between the optical crystallographic data of the hydrates showing the same repeat lattices. A future communication concerning this problem is planned.

**Solubility of Ouabain in Alkanol-Water Systems.**—The solubility of ouabain in ethanol-water solutions and isopropanol-water solutions at 25° was determined and shown in Fig. 2. At 25° ouabain was found to be 1.34% soluble in water. The solubility recorded in The Merck Index is 1 Gm. in about 75 ml. of water. The solubility of ouabain in 95% ethanol is greater than 1% which is reported in the Merck Index. The maximum solubility of ouabain is 3.51% in 42% w/w ethanol-water and 2.7% in 38.42% w/w isopropanol-water solutions.

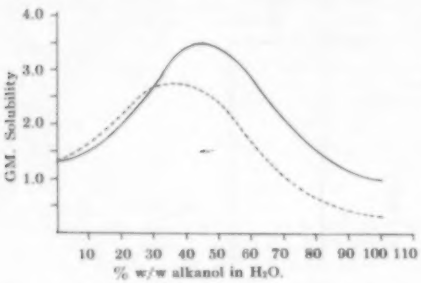



Fig. 2.—Solubility of anhydrous ouabain in ethanol-water (———) and isopropanol-water (- - - -) solutions at 25°.

Recently Moore (17) discussed the relationship of maximum solubility in blended solvents and the dielectric constant. His work was applied to this study. At 25° the dielectric constants for ethanol, isopropanol, and water are, respectively, 24.3, 18.3, and 78.54. Assuming an ideal solution, the dielectric constant for a 42% w/w ethanol in water is 55.76 and the dielectric constant for a 38.42% w/w isopropanol in water is 55.38. This indicates that maximum solubility of ouabain in a blended solvent occurs when the dielectric constant for that solvent is 55.38–55.76.

## SUMMARY

Six different hydrates of ouabain have been isolated. The temperature-water composition phase diagram is reported to describe the phases.

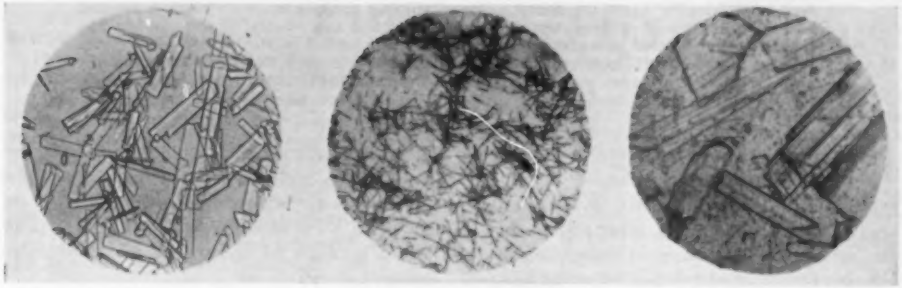
The optical crystallographic properties of the ouabain hydrates have been determined and compared with values reported in the literature for some of the hydrates. The anhydrous ouabain is isotropic.

The *d*-distances for the different hydrates were determined using powders and using copper radiation filtered with nickel. The X-ray powder patterns for ouabain·$4\frac{1}{2}H_2O$ (from ethanol) and ouabain·$4H_2O$ (from methanol) are the same. The X-ray powder patterns for ouabain·$2H_2O$ and the dihydrate which lost 8.12 per cent water on drying at 150° for four hours are the same. It is possible that the optical crystallographic

TABLE III.—THE *d*-DISTANCES FOR THE OUABAIN HYDRATES USING CU K-ALPHA RADIATION

| Ouabain $2H_2O$ | Ouabain·$2H_2O$ Dried 4 Hr. at 130°, Abderhalden | Ouabain $3H_2O$ | Ouabain $4H_2O$ | Ouabain $4\frac{1}{2}H_2O$ | Ouabain $8H_2O$ |
|---|---|---|---|---|---|
| 13.80 | 13.70 | 11.53 | 18.39 | 14.02 | 13.69 |
| 10.21 | 10.27 | 9.60 | 14.24 | 11.11 | 11.04 |
| 8.93 | 9.06 | 8.42 | 11.04 | 9.21 | 10.21 |
| 7.86 | 7.93 | 7.33 | 9.16 | 7.52 | 9.25 |
| 6.68(3) | 6.75(3) | 6.70 | 8.26 | 6.63(1) | 8.30(1) |
| 6.17(2) | 6.19(2) | 6.34(1) | 7.49 | 6.17 | 7.62 |
| 5.75 | 5.80 | 5.79 | 6.68(1) | 5.90(2) | 6.99 |
| 5.18 | 5.17 | 5.40(2) | 6.15 | 5.71 | 6.73(2) |
| 4.87(1) | 4.88(1) | 4.90 | 5.90(2) | 5.09(3) | 6.17 |
| 4.38 | 4.58 | 4.42(3) | 5.50 | 4.87 | 5.59 |
| 4.16 | 4.37 | 3.89 | 5.01(3) | 4.70 | 5.16(3) |
| 3.71 | 4.16 | 3.63 | 4.77 | 4.50 | 4.82 |
| 3.40 | 4.00 | 3.45 | 4.50 | 4.27 | 4.55 |
| 3.09 | 3.85 | 3.17 | 4.32 | 4.05 | 4.27 |
| 2.63 | 3.73 | 4.01 | 4.08 | 3.73 | 4.02 |
| 2.38 | 3.40 | 2.88 | 3.93 | 3.52 | 3.83 |
| | 3.27 | 2.79 | 3.70 | | 3.56 |
| | 3.08 | 2.70 | 3.54 | | 3.42 |
| | 2.89 | 2.54 | 3.42 | | |
| | 2.71 | 2.36 | 3.26 | | |
| | 2.61 | 2.26 | 2.07 | | |
| | 2.53 | 2.21 | 2.92 | | |

OUABAIN·$9H_2O$ OUABAIN·$8H_2O$ OUABAIN·$4^1/_2H_2O$



OUABAIN·$4H_2O$ OUABAIN·$3H_2O$ OUABAIN·$2H_2O$

OUABAIN·$2H_2O$

Fig. 3.—Photomicrographs showing crystal habits of ouabain hydrates, dried at 150° for four hours. All magnifications ×40.

properties distinguish different solvated phases of ouabain to which the X-ray powder patterns are insensitive. The entrapped water is evidently not a part of the repeat lattice but held by hydrogen bonding. The entrapped water is held tightly since the hydrates are stable at room temperature.

Photomicrographs of the ouabain hydrates are shown in Fig. 3. The close similarities of the phases are easily demonstrated with the photomicrographs.

The solubility of the ouabain in ethanol-water and isopropanol-water solutions have been reported. It was concluded that ouabain is most soluble in blended solvents having a dielectric constant of 55.38–55.76 at 25°.

## REFERENCES


(1) Schwartze, E. W., Hann, R. M., and Keenan, G. L., *J. Pharmacol. Exptl. Therap.*, **36**, 481(1929).
(2) Keenan, G. L., THIS JOURNAL, **38**, 355(1949).
(3) Shell, J. W., and Witt, N. F., *ibid.*, **42**, 755(1953).
(4) Kofler, A., and Kofler, L., *Monatsh.*, **80**, 555(1949).
(5) Mannich, C., and Siewart, G., *Ber.*, **75**, 737(1942).
(6) Arnaud, C. R., *Acad. sci. Paris*, **107**, 1011(1888).
(7) Richaud, A., *J. pharm. chim.*, **24**, 161(1921).
(8) "United States Pharmacopeia," 15th Rev., Mack Printing Co., Easton, Pa., 1955.
(9) "The Merck Index," 6th ed., Merck and Co., Inc., Rahway, N. J., 1952, p. 710.
(10) Arnaud, C. R., and Tiffeneau, M., *Bull. acad. med. Paris*, **85**, 187(1921).
(11) Holste, A., *Z. exptl. Pathol. Therap.*, **19**, 153(1918).
(12) Haskell, C. C., and Doeppers, W. A., THIS JOURNAL, **3**, 646(1914).
(13) Luhr, W., and Rietschel, H. G., *Pharm. Zentralhalle*, **80**, 409, 2126(1939).
(14) Berry, H., *Quart. J. Pharm. and Pharmacol.*, **8**, 464(1935).
(15) Biles, J. A., Witt, N. F., and Poe, C. F., THIS JOURNAL, **42**, 53(1953).
(16) "International Critical Tables," Vol. 7, McGraw-Hill Book Co., Inc., New York, N. Y., 1930, pp. 66–67.
(17) Moore, W. E., THIS JOURNAL, **47**, 855(1958).

# Formation of Complexes By N,N′-Dimethyl-diketobenzodiazines in Nonaqueous Solutions*

By AMIN F. HADDAD†, BARTLEY J. SCIARRONE‡, and TAKERU HIGUCHI

**A study has been made of the complexing behavior in carbon tetrachloride of three N,N′-dimethyl-diketobenzodiazines with phenols. 1,3-Dimethylbenzoyleneurea in carbon tetrachloride forms insoluble complexes with hydroquinone and resorcinol, and a soluble complex with phenol. Catechol, on the other hand, forms, at low concentrations, a soluble complex; and then an insoluble complex starts to form after the solubility limit of catechol is reached. 2,3-Diketo-1,4-dimethylquinoxaline in carbon tetrachloride has a tendency to form soluble complexes with resorcinol and favors formation of various complexes with catechol and phenol of the soluble and insoluble types. No complexing tendency was observed in the case of hydroquinone. Within the concentration limits of the phenols used, no complex formation was observed in the systems containing 2,3-dimethylphthalylhydrazide and catechol, resorcinol, or hydroquinone; phenol, however, formed a soluble complex.**

IN RECENT YEARS a large number of studies concerned with complex formation in aqueous systems have been reported. Many of these studies were designed in an attempt to elucidate the nature of the bonding forces responsible for complex formation and to determine the chemical and physical properties of these so-called molecular complexes.

The nature of these forces, especially in aqueous solutions, is still somewhat in doubt. However, it is generally believed that the main forces responsible for complex formation are basically electrostatic in nature and that the particular type of the interaction force or forces involved may be of various kinds depending on the component entering into the formation of the individual complex molecule.

In explaining complex formation in aqueous solutions, Higuchi and Lach (1) pointed out that on the basis of electrostatic attraction between the interacting groups alone the extent of complex formation would be extremely small since the competing water dipoles are present in such high concentration. They believe that the tendency for complex formation in such a system is rendered favorable by the interaction of the hydrophobic, lipophilic portions of the component molecules in a manner similar to micelle formation. These hydrophobic units are thought to be "squeezed out" of the water phase by the high internal pressure of water leading to a high degree of interaction. This is explained by the same authors, on the basis that the squeezing together of the hydrocarbon portions of the reactants permits the water molecules originally surrounding these units to form additional hydrogen bonds; the net effect is an apparent high degree of interaction between the relatively inert hydrocarbon groups.

Earlier works (1–4) indicated that caffeine is particularly capable of forming molecular complexes with pharmaceutical acids, esters, salts, and certain aromatic and nonaromatic nitrogenous compounds. The complexing behavior of caffeine has been attributed (4) to the carbonyl group activated by the adjacent nitrogen atom. Consequently in an attempt to provide a better understanding of the intermolecular forces involved in the formation of molecular complexes and the structural requirements favoring complex formation in aqueous systems, a series of compounds having structural similarities to caffeine have been chosen and their complexing tendencies are being studied (9) in aqueous solutions using phenolic substances as the complexing agent. Results of these studies will be reported at a later date. The present study is concerned with the complexing behavior of three of these compounds in a nonaqueous media in order to eliminate the possibility of the occurrence of the competitive reactions, phenol-substrate and water-substrate interactions which are believed to exist in aqueous systems due to the high polarity of water. Accordingly carbon tetrachloride has been chosen as the nonpolar solvent in the present preliminary study. It is hoped that through the use of nonpolar solvents, the solute-solvent interactions might be eliminated, thus permitting a better understanding of the intramolecular and intermolecular forces existing between the components of a molecular complex formed both in aqueous and nonaqueous solutions.

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## EXPERIMENTAL

**Reagents.**—Phenol, analytical reagent grade. Resorcinol, U. S. P. recrystallized. Hydroquinone, photo-purified, Mallinckrodt. Catechol, resublimed, m. p. 104.5–106.5°. 1,3-Dimethylbenzoyleneurea: prepared in the laboratory by the methylation of benzoyleneurea with dimethylsulfate in an alkaline medium, m. p. 164–166°. Benzoyleneurea was prepared by the process of Bogert and Scatchard (5), slightly modified. 2,3-Diketo-1,4-dimethylquinoxaline: 2,3-diketoquinoxaline was first prepared (6) and then methylated by using dimethylsulfate in an alkaline solution (7), m. p. 254–256°. 2,3-Dimethylphthalylhydrazide was prepared (8) by the condensation of phthalic anhydride and symmetrical dimethylhydrazine, m. p. 175–177°. Carbon tetrachloride, analytical reagent grade.

**Procedure.**—The solubility method was employed in determining the extent and nature of possible complex formation in the systems under investigation. The substrate and the complexing agents, except phenol, were accurately weighed in the dry state into 10-ml. Kimble clear-glass ampuls. Ten milliliters of carbon tetrachloride were then added. In the case of phenol, accurately measured volumes of a standard solution were added to the substrate and the final volume was made up to 10 ml. by the addition of the required volume of carbon tetrachloride. The hermetically sealed ampuls were immersed in a constant temperature water bath equipped with a rotating bar to which the racks, holding ten ampuls each, were fastened. The ampuls were allowed to equilibrate at 30° for periods ranging from forty to forty-six hours.

After equilibration the ampuls were opened and the required volume of solution was withdrawn by means of a volumetric pipet fitted with a piece of latex-rubber tubing enclosing a plug of glass wool as filter. In the case of 2,3-diketo-1,4-dimethylquinoxaline, a layer of glass powder between the two glass wool layers was necessary to filter off the colloidal precipitate. The resulting clear solution was suitably diluted with carbon tetrachloride to the required volume in volumetric flasks and the final dilution analyzed on Model 11 ms Cary recording spectrophotometer. In order to eliminate the interference of the phenols, which start absorbing at about 310 mμ wavelength, the absorbance values of the substrate were read at 324 mμ wavelength.

## RESULTS AND DISCUSSIONS

**1,3-Dimethylbenzoyleneurea.**—The complexing tendencies of this compound with catechol, resorcinol, hydroquinone, and phenol were studied in carbon tetrachloride. The results are reported here below.

*Catechol.*—The interaction between 1,3-dimethylbenzoyleneurea and catechol is shown in Fig. 1. In order to build up the phase diagram five runs were carried, each on a different day. The phase diagram shows that at low catechol concentration a straight line relationship is obtained between the dimethylbenzoyleneurea and catechol. The increase in the solubility of the dimethylbenzoyleneurea is due to the formation of a soluble complex which continues until the solubility limit (about $1.379 \times 10^{-2}$ moles per liter) of catechol is reached.

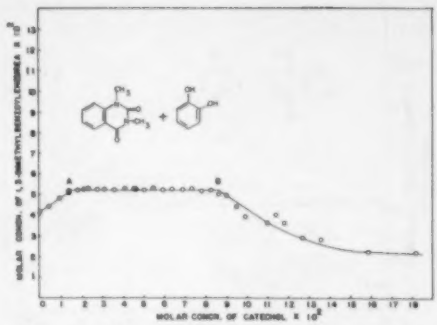


Fig. 1.—Phase solubility plot showing interaction between 1,3-dimethylbenzoyleneurea and catechol in carbon tetrachloride.

Point A in Fig. 1 represents the saturation point of the solution with regard to the complex. Further addition of catechol results in formation of more complex which precipitates from the already saturated system. The precipitation continues until the excess of the dimethylbenzoyleneurea added to the system has been utilized and the solubility curve begins to descend at point B. This drop is due to the complete consummation of all excess dimethylbenzoyleneurea and subsequent depletion of this material in solution.

The stoichiometric ratio of the components of the complex which was formed in the plateau region can be calculated from the phase diagram. This is possible because the excess of free dimethylbenzoyleneurea found at point A is equivalent to the amount being converted to the complex during the interval between points A and B in Fig. 1. The corresponding amount of the catechol being converted to the complex is equal to that entering into the complex during the same interval.

Calculations:

Dimethylbenzoyleneurea content of complex formed in the plateau region = total dimethylbenzoyleneurea added to system − dimethylbenzoyleneurea in solution at point A = $7.97 \times 10^{-2} - 5.22 \times 10^{-2} = 2.75 \times 10^{-2}$

Catechol content of complex in the same region = $8.62 \times 10^{-2} - 1.45 \times 10^{-2}$ (read from diagram) = $7.17 \times 10^{-2}$

Then the stoichiometric ratio $= \frac{\text{Dimethylbenzoyleneurea}}{\text{catechol}}$

$$= \frac{2.75 \times 10^{-2}}{7.17 \times 10^{-2}}$$

$$= \frac{1}{2.6} \text{ or } \frac{2}{5.12}$$

The above results indicate that, most probably, the complex formed is a 2:5 complex. According to the phase diagram other complexes are possible.

*Resorcinol.*—The phase diagram in Fig. 2 indicates that there is a definite interaction between 1,3-dimethylbenzoyleneurea and resorcinol. There is a very slight initial rise in the curve, which could be attributed to experimental error, indicating that the complex formed is extremely insoluble in carbon tetrachloride, since no significant initial increase in the apparent solubility of dimethylbenzoyleneurea is observed, in contrast to the dimethylbenzoyleneurea-

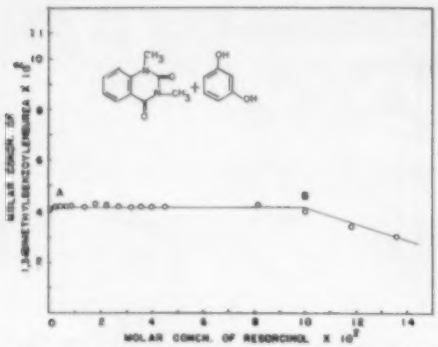


Fig. 2.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and resorcinol in carbon tetrachloride.

catechol system discussed above. It is worth mentioning here that resorcinol and hydroquinone are extremely insoluble in carbon tetrachloride as compared to catechol, which is soluble to the extent of about $1.379 \times 10^{-2}$ moles/L. Phenol is very soluble in carbon tetrachloride. This difference in the solubility behavior of these phenols in carbon tetrachloride might explain the difference in the solubility behavior of the corresponding complexes formed.

The ratio of the components of the complex formed in the plateau region can be calculated from the plot in Fig. 2. This is possible because the plateau region represents the amount of resorcinol entering into complex formation during this interval and the corresponding amount of dimethylbenzoyleneurea being converted to the complex. The amount of the latter component is equal to that present in the form of free undissolved solid in the system at zero resorcinol concentration. The system between points A and B is invariant, which means that as the dimethylbenzoyleneurea is removed in the form of insoluble complex it is replaced from the excess present in the system. When all the excess of dimethylbenzoyleneurea is used up the dimethylbenzoyleneurea titer drops as shown in the diagram at point B. Therefore, by determining the quantities of resorcinol and dimethylbenzoyleneurea used in the plateau region, the stoichiometry of the complex formed during the same interval can be calculated.

Calculations:

Dimethylbenzoyleneurea content of complex formed in the plateau region = total dimethylbenzoyleneurea added to system − dimethylbenzoyleneurea in solution at point A = $6.75 \times 10^{-2} - 4.17 \times 10^{-2} = 2.58 \times 10^{-2}$

Resorcinol content of complex during same interval = $10.035 \times 10^{-2}$

$$\text{Then the stoichiometric ratio} = \frac{\text{dimethylbenzoyleneurea}}{\text{resorcinol}}$$

$$= \frac{2.58 \times 10^{-2}}{10.035 \times 10^{-2}} \text{ or roughly } \frac{1}{4}$$

*Hydroquinone.*—This compound is extremely insoluble in carbon tetrachloride. The phase diagram in Fig. 3 shows a weak binding activity between hydroquinone and dimethylbenzoyleneurea. From the phase diagram it is possible to calculate for the ratio of the components of the complex formed in the plateau by employing the same procedure described above under the dimethylbenzoyleneurea-resorcinol system.

Calculation:

$$\frac{\text{Dimethylbenzoyleneurea content of the complex formed in the plateau region}}{\text{Hydroquinone content of complex in the same region}} = \frac{6.84 \times 10^{-2} - 4.05 \times 10^{-2}}{3.67 \times 10^{-2}}$$

$$= \frac{2.79 \times 10^{-2}}{3.67 \times 10^{-2}} = \frac{1}{1.32}$$

*Phenol.*—The influence of phenol on the solubility of 1,3-dimethylbenzoyleneurea is shown in Fig. 4. The diagram shows that dimethylbenzoyleneurea and phenol form a soluble complex in the presence of zero to $5.334 \times 10^{-2}$ molar concentration used of the latter component. The molecular ratio of the complex cannot be calculated. Assuming a 1:1 stoichiometric ratio the value of the stability constant of this system is 2.44.

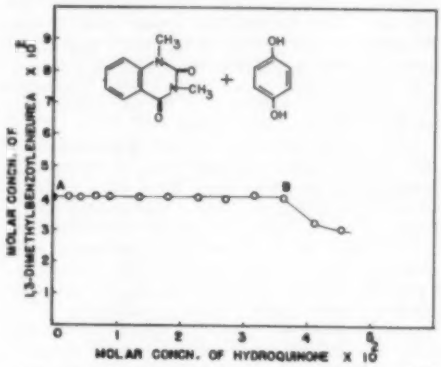


Fig. 3.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and hydroquinone in carbon tetrachloride.

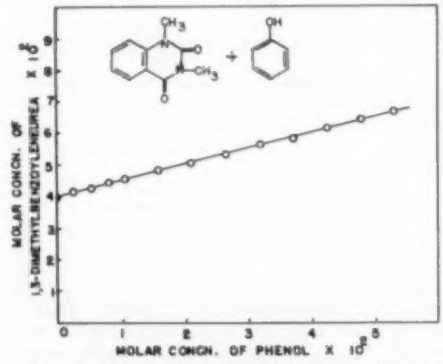


Fig. 4.—Phase solubility plot of 1,3-dimethylbenzoyleneurea and phenol in carbon tetrachloride.

**2,3-Diketo-1,4-dimethylquinoxaline.**—A study was carried out on the possibility of complex formation in systems containing 2,3-diketo-1,4-dimethylquinoxaline and catechol, resorcinol, hydroquinone, and phenol. The following is a brief discussion of the results.

*Catechol.*—It was observed that 2,3-diketo-1,4-dimethylquinoxaline has a tendency to form with catechol several forms of molecular complexes of the soluble and the insoluble types. No diagram of this system is reproduced in the present communication.

*Resorcinol.*—This compound forms a soluble complex with 2,3-diketo-1,4-dimethylquinoxaline as is shown in Fig. 5. It is significant to note the high proportion of resorcinol consumed for every unit rise in the solubility of the quinoxaline compound.

*Hydroquinone.*—No complexing tendency was observed in the system containing hydroquinone and 2,3-diketo-1,4-dimethylquinoxaline.

*Phenol.*—The phase diagram shown in Fig. 6 indicates that systems containing phenol and 2,3-diketo-1,4-dimethylquinoxaline favor formation of various forms of complexes of the soluble and the insoluble types dependent upon the molar concentration of phenol present in the system.

**2,3-Dimethylphthalylhydrazide.**—Within the concentration limits of the phenols used no complex formation was observed in the systems containing

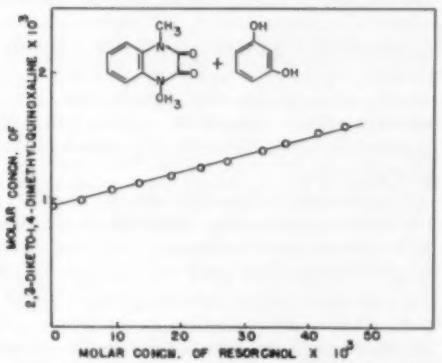


Fig. 5.—Phase solubility plot of diketodimethylquinoxaline and resorcinol in carbon tetrachloride.

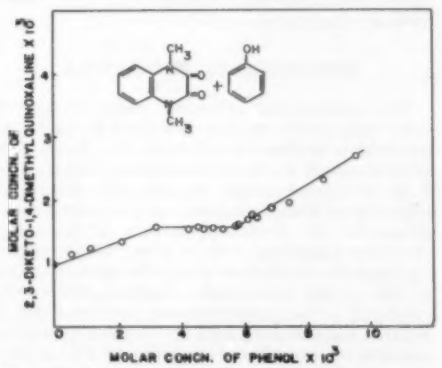


Fig. 6.—Phase solubility plot of diketodimethylquinoxaline and phenol in carbon tetrachloride.

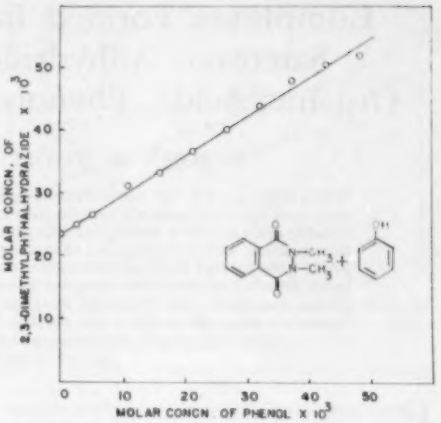


Fig. 7.—Phase solubility plot of dimethylphthalylhydrazide and phenol in carbon tetrachloride.

2,3-dimethylphthalylhydrazide and catechol, resorcinol, and hydroquinone. Phenol, however, has a tendency to form soluble molecular complexes with the phthalylhydrazide compound as shown in Fig. 7.

## CONCLUSION

The experimental findings presented above show that, of the three, N, N′-dimethyl-diketobenzodiazines studied, 1,3-dimethylbenzoyleneurea has the strongest tendency to form complexes in carbon tetrachloride with catechol, hydroquinone, resorcinol, and phenol. 2,3-Diketo-1,4-dimethylquinoxaline forms complexes with catechol, resorcinol, and phenol but not with hydroquinone. On the other hand, 2,3-dimethylphthalylhydrazide forms complexes with phenol, but no complexes were observed with catechol, hydroquinone, and resorcinol.

Although the experimental findings of this preliminary study are insufficient to elucidate the nature of the structural relationship and binding forces existing between the components of the molecular complexes, they do indicate that complex formations in the systems studied are possible in nonaqueous solvents where the competitive forces manifested by water in aqueous systems are absent.

## REFERENCES

(1) Higuchi, T., and Lach, J. L., THIS JOURNAL, **43**, 349, 465, 524(1954).
(2) Pellini, G., *Atti accad. nas. Lincei*, **19**, 329(1910).
(3) Zuck, D. A., Thesis for Ph.D., University of Wisconsin, 1952.
(4) Higuchi, T., and Zuck, D. A., THIS JOURNAL, **42**, 132 (1953).
(5) Bogert, M. T., and Scatchard, G., *J. Am. Chem. Soc.*, **38**, 1611(1916).
(6) Philips, M. A., *J. Chem. Soc.*, **1928**, 2393.
(7) Newbold, G. T., and Spring, F. S., *ibid.*, **1948**, 519.
(8) Drew, H. D. K., Hatt, H. H., and Hobart, F. A., *ibid.*, **1937**, 33.
(9) Sciarrone, B. J., Thesis Project, University of Wisconsin.

# Complexes Formed in Aqueous Solutions By Sarcosine Anhydride; Interactions With Organic Acids, Phenols, and Aromatic Alcohols*

By JOHN W. POOLE† and TAKERU HIGUCHI

**It has been shown that the interactions between sarcosine anhydride, a cyclic dipeptide, and forty-six organic proton donor compounds result in the formation of both soluble and insoluble molecular complexes. The studies illustrate the importance of the aromatic and hydrophobic character of the molecule, as well as the nature of the polar groups and their positions, on complexing activity. The stoichiometry of a large number of insoluble complex species found were determined from analysis of phase diagrams and chemical analysis of the solid materials. Apparent stability constants were determined for the soluble complexes formed in the investigation. It is suggested that many biochemical and drug actions are mediated through formation of complexes of this type.**

PREVIOUS INVESTIGATIONS (1–10) have demonstrated the formation of molecular complexes in aqueous solutions by the interaction of compounds capable of donating protons with such agents as caffeine, polyethylene glycols, polyvinylpyrrolidone, and a series of water-soluble amides. The present study is concerned with similar complex formation between sarcosine anhydride and various acidic substances which were chosen to illustrate the structural factors favoring these interactions. The complexing tendencies of various acids, phenols, and alcohols with sarcosine anhydride were observed for this purpose by means of solubility studies. In addition, where insoluble complexes were formed the stoichiometric ratios and melting points of these products were determined.

Ability of sarcosine anhydride to form various molecular association compounds in aqueous solution was thought to be of particular pharmaceutical interest for two reasons. It is, in a sense, a dipeptide which may be expected to present in aqueous phase a surface crudely comparable to that of a protein (enzyme). As has been previously speculated, interactions of this type may well mediate drug and biochemical reactions. Secondly, because of its amino acid origin, sarcosine anhydride may prove to be an acceptable nontoxic complexing agent useful in modifying solubility and stability properties of certain drugs from a formulation standpoint.

Specifically, the relative tendency of sarcosine anhydride to form molecular complexes with the following compounds were determined: *ortho*-, *meta*-, and *para*-hydroxy benzoic acids, benzoic acid, aspirin, *ortho*-phthalic acid, 4-aminosalicylic acid, 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, gallic acid, 8-hydroxyquinoline, gallacetophenone, phloroglucinol, 2,5-dihydroxybenzoic acid ethanolamide, 2,3-dihydroxyquinoxaline, *ortho*-, *meta*-, and *para*-aminophenols, salicylamide, salicyl alcohol, salicylamine, 1,5-naphthalenediol, 2,7-naphthalenediol, hydroquinone, 2,6-dihydroxytoluene, hexylresorcinol, *ortho*-, *meta*-, and *para*-xylylene glycols, suberic acid, pimelic acid, adipic acid, rutin, oxytetracycline, melamine, chloramphenicol, quinine hydrochloride, picric acid, phenobarbital, and L-tyrosine.

The purpose of this study was (*a*) to observe the tendency of sarcosine anhydride to associate with proton-donor compounds with the possibility of interpreting these data in terms of the stereochemical factors favoring complex formation; (*b*) to note whether a compound like sarcosine anhydride, with little aromatic character, would be capable of entering into any strong specific interactions; and (*c*) to obtain general information useful in the elucidation of the whole field of complexing.

## EXPERIMENTAL PROCEDURE

The experimental procedure employed in this study was similar to that described in an earlier publication by Higuchi and Lach (6). Small glass vials were used as the reaction vessels with 10, 5, or 1 ml. of solvent added, depending on the initial solubility and/or availability of the proton-donor substance. A spectrophotometric analysis was utilized in a majority of the studies. In the remaining cases, the interactions were followed by titration of the acidic compound. Distilled water was employed as the general solvent, however 0.001 *N* sulfuric acid was used with the carboxylic acids to suppress any ionization of the acids. A 20% sodium chloride solution was utilized with *meta*-xylylene glycol to substantially reduce the initial solubility of

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† Fellow of The American Foundation for Pharmaceutical Education.

this compound. When the donor substance was especially labile to oxidation, 0.1% sodium bisulfite was added to the system.

The relationship between concentration and volume fraction of sarcosine anhydride was obtained from specific gravity measurements. The specific gravities of several sarcosine anhydride solutions were determined by the pycnometer method.

All of the compounds employed in this investigation were of reagent grade or recrystallized from suitable solvents.

**Synthesis of Sarcosine Anhydride.**—Sarcosine anhydride, 1,4-dimethyl-1,5-diketopiperazine, was prepared essentially by the method described by Bilek, *et al.* (11), for the preparation of N,N′-diethyl-2,5-diketopiperazine. The ethyl ester of sarcosine was obtained by treating sarcosine hydrochloride with absolute ethanol and dry HCl gas. The resulting ester hydrochloride was transformed to the free ester by the use of anhydrous ammonia and separated by vacuum distillation. The ethyl ester of sarcosine was placed in a sealed tube and heated for thirty-six hours at 180°. The product was purified by recrystallization from absolute ethyl alcohol with overall yields of 30–40%. The recrystallized compound has m. p. 145–146°, reported (12) m. p. 149°.

## RESULTS AND OBSERVATIONS

**General Behavior.**—In a majority of the cases a straight line is obtained when the apparent solubility of the acidic compound in water is plotted against the amount of sarcosine anhydride present. The phase diagrams for the interactions of sarcosine anhydride with the aminobenzoic acids, shown in Fig. 1, are typical for this type system. The principal complex being formed, if we attribute the increase in solubility to complex formation, is then directly dependent on the sarcosine anhydride concentration in these ranges. Because the concentration of the free acid substance remains constant, the stoichiometric ratio of the complexes formed in these studies cannot be determined from the phase diagrams. The only requirement for the straight line plot is for the complex formed to be unimolecular with respect to the sarcosine anhydride. It is evident that if the stoichiometry of these complexes is unknown the true stability constants cannot be calculated. If some stoichiometry is assumed, however, the apparent stability constants can be evaluated by use of Eq. 1.

$$K_m = \frac{(A_nB)}{(A)^n(B)} \qquad \text{(Eq. 1)}$$

where $(A_nB)$ is the concentration of complex; $(A)$ is the concentration of free acidic substance; $(B)$ is the concentration of free sarcosine anhydride; and $n$ is the number of molecules of acidic substance associated with each sarcosine anhydride molecule. The details for this calculation have been shown by Higuchi and Zuck in a previous publication (3).

The stability constants based on the assumption of a 1:1 molecular complex were calculated for nearly all of the systems investigated to facilitate a comparison of the complexing tendency of sarcosine anhydride with similar complexing agents previously studied, as well as to give an indication of the order of complexing activity demonstrated by the substances studied. These constants are included in Tables I, III, V, VI, and VII.

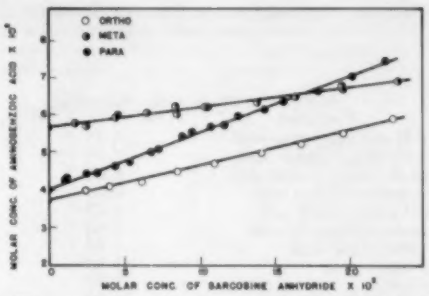


Fig. 1.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of the aminobenzoic acids in water at 25°.

Since the majority of insoluble complexes obtained in this investigation had a stoichiometric ratio of two acid molecules to one molecule of sarcosine anhydride, it is reasonable to expect a predominance of a 2:1 molecular ratio in the soluble complexes formed in solution. The stability constants calculated on this basis appeared to give a more accurate description of the overall complexing ability of sarcosine anhydride. The constants determined on this basis are also included in the tables previously mentioned. Since there is a possibility of several simultaneous equilibria in solution, the nature of the interaction cannot be accurately described.

Plateau regions in the phase diagram were found with substances which formed more insoluble complexes. This invariant region is a result of the solution being saturated with respect to both the complex and the acidic compound. The interaction between limited excess of 2,5-dihydroxybenzoic acid and sarcosine anhydride, for example, results in an insoluble complex, which is illustrated by the phase diagram in Fig. 2. The stoichiometry of complexes of this type can be calculated from the phase diagrams according to the method of Higuchi and Lach (6). Thus, it can be seen from Fig. 2 that the concentration of acid in solution remains constant along the line AB. At point B all the excess solid acid has been precipitated as complex, beyond this point 2,5-dihydroxybenzoic acid is being depleted from solution. The amount of sarcosine anhydride repre-

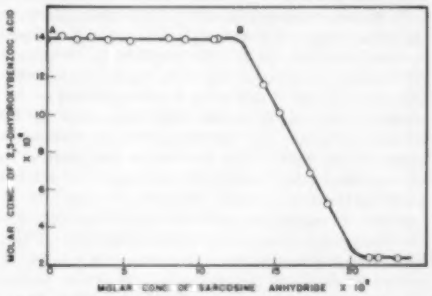


Fig. 2.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,5-dihydroxybenzoic acid in water at 25°. The system contained a total of 0.390 mole/L. of 2,5-dihydroxybenzoic acid in and out of solution.

TABLE I.—INTERACTION OF VARIOUS AROMATIC CARBOXYLIC ACIDS WITH SARCOSINE ANHYDRIDE AT 25°

| Compound | Orig. Solubility, Molar Concn. × $10^2$ | $K_1$[a], (liter) (mole) | $K_1$[b] (liter) (mole) | Increase in Solubility With a 1% Sarcosine Anhydride Soln., % |
|---|---|---|---|---|
| *p*-Hydroxybenzoic acid | 4.31 | 5.4 | 54[c] | 30 |
| *o*-Hydroxybenzoic acid | 1.75 | 4.3 | 150 | 30 |
| *m*-Hydroxybenzoic acid | 5.85 | 3.6 | 27[d] | 21 |
| *p*-Aminobenzoic acid | 4.03 | 4.2 | 49 | 26 |
| *o*-Aminobenzoic acid | 3.73 | 2.6 | 34 | 17 |
| *m*-Aminobenzoic acid | 5.63 | 1.1 | 10 | 7 |
| Isonicotinic acid | 4.73 | 0 | 0 | 0 |
| Benzoic acid | 2.85 | 2.9 | 50 | 19 |
| Aspirin | 2.27 | 3.1 | 65 | 19 |
| *o*-Phthalic acid | 3.90 | 2.2 | 29 | 16 |
| 4-Aminosalicylic | 1.00 | 5.1 | 250 | 36 |
| 2,4-Dihydroxybenzoic acid | 3.75 | 3.4 | 63[e] | .. |
| 3,4-Dihydroxybenzoic acid | 8.92 | 9.6 | 38[f] | .. |
| 2,5-Dihydroxybenzoic acid | 13.6 | .... | .. | .. |
| 3,5-Dihydroxybenzoic acid | 66.0 | .... | .. | .. |
| Gallic acid | 7.78 | 64 | .. | .. |

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis, determined from one point.
[c] Value from downcurve is 51.
[d] Value from downcurve is 25.
[e] Value from downcurve is 66.
[f] Value from downcurve is 40.

sented by the length of the plateau is equal to that entering into the insoluble complex during that interval. The difference between the total 2,5-dihydroxybenzoic acid added to the system and that found in solution at point A is the corresponding amount of acid converted to the insoluble complex.

Thus,

sarcosine anhydride content of complex formed in the plateau region = $12.6 \times 10^{-2}$ moles/L. (from diagram)

2,5-dihydroxybenzoic acid content = total acid-acid in solution at point A
$= 39.0 \times 10^{-2} - 14 \times 10^{-2}$
$= 25.0 \times 10^{-2}$ moles/L.

Then the stoichiometric ratio equals 2,5-dihydroxybenzoic acid content $= \frac{25.0 \times 10^{-2}}{12.6 \times 10^{-2}} = 2.0$

The results indicate that two molecules of acid are associated with each molecule of sarcosine anhydride.

In those instances where the descending portion of the phase diagram is a straight line extending closely to the base line, it is also possible to obtain the stoichiometric ratio of the complex in the following manner. If the downcurve is extrapolated to zero concentration of the acidic substance, then the difference between the concentration of complexing agent at the start of the downcurve and that at the extrapolated point, minus the amount of complexing agent present as soluble complex, is equal to the amount of complexing agent needed to interact with the free proton-donor substance in solution. Therefore, the stoichiometry of the complex can be obtained by dividing the concentration of the proton-donor compound in solution by the concentration of complexing agent needed to interact with the donor substance in solution. It is possible to demonstrate this calculation for the interaction described by the phase diagram in Fig. 2.

concentration of sarcosine anhydride at extrapolated point = $20.x \times 10^{-2}$ moles/L.

concentration of sarcosine anhydride at start of downcurve = $12.6 \times 10^{-2}$ moles/L.—sarcosine at extrapolated point anhydride at start of downcurve—sarcosine anhydride present as soluble complex
$= 20.2 \times 10^{-2} - 12.6 \times 10^{-2}$
$= 7.6 \times 10^{-2}$ moles/L.

2,5-dihydroxybenzoic acid in solution = $14.0 \times 10^{-2}$ moles/L.

Then the stoichiometric ratio equals 2,5-dihydroxybenzoic acid content/sarcosine anhydride content $= \frac{14.0 \times 10^{-2}}{7.6 \times 10^{-2}} = 1.8$

The results indicate that the reaction product is probably a two-to-one complex.

The stoichiometric ratios obtained by analysis of the phase diagrams were confirmed by isolation and chemical analysis of the insoluble reaction products. Tables II and IV list the stoichiometric ratios determined by the various methods along with the melting points of the insoluble complexes.

Where the plateau region of the phase diagram is preceded by a rise in the curve, as exhibited in the interactions of sarcosine anhydride with *meta*- and *para*-hydroxybenzoic acids shown in Fig. 4, it is possible to obtain the stability of the complex formed. For interactions of this type the descending portion of the curve can also be used to determine the stability constant by use of Eq. 1 in the following manner:

The complex in solution, ($A_nB$), is determined from the difference between the original solubility of the acidic substance and the maximum concentration it attains at the plateau; (B), the free complexing agent in solution = (total complexing agent

TABLE II.—STOICHIOMETRIC RATIOS AND MELTING POINTS OF THE INSOLUBLE COMPLEXES FORMED BETWEEN AROMATIC CARBOXYLIC ACIDS AND SARCOSINE ANHYDRIDE

| Compound | Molecular Ratio Found of Acid : Sarcosine Anhydride | | | Probable Stoichiometric Ratio | M. p. of Complex, °C. |
|---|---|---|---|---|---|
| | Plateau[a] | Extrapolation[b] | Chem. Anal.[c] | | |
| *m*-Hydroxybenzoic acid | 1.9:1 | ... | 2.2:1 | 2:1 | 154–156 |
| *p*-Hydroxybenzoic acid | 2.4:1 | ... | 2.4:1 | 2:1 | 188–190 |
| 2,4-Dihydroxybenzoic acid | 2.1:1 | ... | 1.8:1 | 2:1 | 187–188 |
| 2,5-Dihydroxybenzoic acid | 2.0:1 | 1.8:1 | 1.8:1 | 2:1 | 183–184 |
| 3,4-Dihydroxybenzoic acid | 2.2:1 | 2.2:1 | 1.9:1 | 2:1 | 188–192 |
| 3,5-Dihydroxybenzoic acid | 5.9:1 | 5.6:1 | 6.3:1 | 6:1 | 212–220 |
| Gallic acid | 6.0:1 | 5.6:1 | 5.2:1 | 6:1 | 210–220 |

[a] Determined from length of plateau.
[b] Determined from extrapolation of the downcurve.
[c] Determined from chemical analysis of the insoluble material.

TABLE III.—INTERACTIONS OF VARIOUS AROMATIC ALCOHOLS AND PHENOLS WITH SARCOSINE ANHYDRIDE AT 25°

| Compound | Orig. Solubility, Molar Concn. × $10^2$ | $K_1$[a] (liter) (mole) | $K_2$[b] (liter) (mole) | Increase in Solubility With a 1% Sarcosine Anhydride Soln. |
|---|---|---|---|---|
| 8-Hydroxyquinoline | 0.41 | 8.2 | 1,000 | 59 |
| Gallacetophenone | 3.38 | 8.3 | 120 | 46 |
| Phloroglucinol | 16.5 | 12 | 18 | 29 |
| 2,5-Dihydroxybenzoic acid ethanolamide | 28.6 | 39 | 10 | 23 |
| 2,3-Dihydroxyquinoxaline | 0.15 | 2.5 | 800 | 18 |
| *o*-Aminophenol | 2.96 | 2.3 | 37 | 15 |
| *p*-Aminophenol | 5.03 | 1.9 | 18 | 15 |
| *m*-Aminophenol | 28.5 | 5.6 | 5.6 | 14 |
| Salicylamide | 1.82 | 3.9 | 110 | 26 |
| Salicyl alcohol | 59.5 | ... | 2.9 | 13 |
| Salicylamine | 7.80 | 0.8 | 4.8 | 5 |
| 1,5-Naphthalenediol | 0.15 | 23[c] | .... | .. |
| 2,7-Naphthalenediol | 2.55 | ... | .... | .. |
| Hydroquinone | 63.5 | ... | .... | .. |
| 2,6-Dihydroxytoluene | 212 | ... | .... | .. |
| Hexylresorcinol | .... | ... | .... | .. |

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.
[c] Value from downcurve is 22.

added)−(amount in soluble complex)−(amount precipitated as complex on the downcurve). When the stability constants were calculated on the basis of the stoichiometry found for the insoluble complexes, the same value for the stability constant was obtained from the rising and descending portions of the curve. The constant was not the same on both sides of the plateau if a 1:1 ratio was assumed when the plateau indicated a 2:1 complex to be the true stoichiometry. Thus, in these cases, it appears that the same reaction is responsible for the rising, descending, and plateau portions of the phase diagram. Since the 2:1 interaction is the one encountered most frequently with the insoluble complexes, it seems reasonable to assume that this is the dominant interaction responsible for many of the soluble complexes. This is especially true for the difunctional benzene derivatives, as they would be analogous to the *meta*- and *para*-hydroxybenzoic acids. The interactions between sarcosine anhydride and salicyl alcohol, orthoxylylene glycol, and *meta*-xylylene glycol also give evidence of a 2:1 stoichiometric ratio. The slopes of the straight lines obtained in these instances are all greater than 1, and it is obvious that there cannot be more sarcosine anhydride in the form of the molecular complex than has been added to the system.

In many cases, the phase diagrams for the interactions resulting in the formation of insoluble complexes exhibit a minimum in the curve at high concentrations of the complexing agent. No attempt has been made to study the interactions responsible for this irregularity, although it is believed to be a result of the formation of more soluble complexes of higher stoichiometric ratios.

The degree of solubilization of the proton-donor compounds was found to relate to the volume fraction of sarcosine anhydride in the system. It can be readily seen from Fig. 3 that the volume fraction

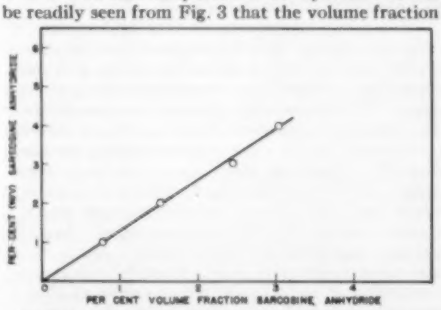


Fig. 3.—Plot showing the relationship of per cent volume fraction of sarcosine anhydride to per cent (w/v) of sarcosine anhydride.

TABLE IV.—STOICHIOMETRIC RATIOS AND MELTING POINTS OF THE INSOLUBLE COMPLEXES FORMED BETWEEN AROMATIC ALCOHOLS AND PHENOLS AND SARCOSINE ANHYDRIDE

| Compound | Molecular Ratio Found of Alcohol or Phenol : Sarcosine Anhydride | | | | |
|---|---|---|---|---|---|
| | Plateau[a] | Extrapolation[b] | Chem. Anal.[c] | Probable Stoichiometric Ratio | M. p. of Complex, °C. |
| Hydroquinone | 2:21 | 1.9:1 | 2.0:1 | 2:1 | 184–185 |
| Hexylresorcinol | .... | .... | 4.9:1 | 5:1 | 76–77 |
| 2,6-Dihydroxytoluene | 2.0:1 | 2.6:1 | 2.2:1 | 2:1 | 170–171 |
| 1,5-Naphthalenediol | 1.1:1 | .... | 1.1:1 | 1:1 | 214–216d |
| 2,7-Naphthalenediol | 2.9:1 | 2.9:1 | 3.0:1 | 3:1 | 171–181 |

[a] Determined from length of plateau.
[b] Determined from extrapolation of the downcurve.
[c] Determined from chemical analysis of the insoluble material.

TABLE V.—INTERACTIONS OF XYLYLENE GLYCOLS WITH SARCOSINE ANHYDRIDE AT 25°

| Compound | Orig. Solubility Molar Concn. × 10² | $K_1$[a] (liter) (mole) | $K_2$[b] (liter) (mole) | Increase in Solubility With a 1% Sarcosine Anhydride Soln., % |
|---|---|---|---|---|
| *m*-Xylylene glycol | 58.5 | .. | 5.2 | 17 |
| *o*-Xylylene glycol | 114 | .. | 5.9 | 11 |
| *p*-Xylylene glycol | 41.1 | 1.4 | 0.1 | 6 |

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.

TABLE VI.—INTERACTIONS OF ALIPHATIC DICARBOXYLIC ACIDS WITH SARCOSINE ANHYDRIDE AT 25°

| Compound | Orig. Solubility, Molar Concn. × 10² | $K_1$[a] (liter) (mole) | $K_2$[b] (liter) (mole) | Increase in Solubility With a 1% Sarcosine Anhydride Soln., % |
|---|---|---|---|---|
| Suberic acid | 0.82 | 2.2 | 120 | 12 |
| Pimelic acid | 28.5 | 1.4 | 1.9 | 9 |
| Adipic acid | 14.8 | 0.5 | 1.7 | 3 |

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.

of sarcosine anhydride is a straight line function of the concentration within the concentration limits of the study. Inasmuch as the solubility curves are also straight lines, it follows that the increase in solubility is related to the volume fraction of complexing agent in the same manner.

The relative complexing activity taking place in these systems can also be compared by comparing the per cent increase in solubility of the acid compounds at some definite concentration of complexing agent. It is interesting, however, to consider first the expected increase in solubility if there were no greater tendency for the acidic substance to associate with the complexing agent than with the solvent water. If this were the case, formation of a complex would result only from a chance nonspecific contact of acid molecules with sarcosine anhydride. In a 1% sarcosine anhydride solution where the volume fraction of the complexing agent is 0.008 there is a rough probability of eight chances in a thousand that a neighbor of an acid molecule would be the complexing agent. Let us assume that such contacting pairs are, at the most, a separate species. Since each acid molecule has, very crudely, ten neighboring molecules in the solution, this probability is increased ten times; and the expected increase in solubility would be roughly 8%, at the most, of the original solubility of the proton-donor substance. Where the increase in solubility is significantly less than this value of 8%, the complex formed may be attributed to a nonspecific solvation effect as opposed to active complexing. The latter must arise from some driving force, which forms complexes in solution, is substantially greater than that predicated by simple statistical expectation, and is obviously present in those interactions which give increases in solubility markedly greater than this calculated value. The per cent increase in the solubility of the electrophilic compounds with a 1% sarcosine anhydride solution are included in Tables I, III, V, VI, and VII.

**Interactions of Sarcosine Anhydride With Various Aromatic Carboxyl Acids.**—This part of the study was undertaken to determine the influence of ring location on formation of molecular complexes in aqueous solution between sarcosine anhydride and various isomeric aromatic acids. The interactions responsible for these complexes would be expected because of the electrophilic and nucleophilic nature of the reactants. Further, the effect of various proton-donating groups on the ability of these acids to form complexes was noted.

The results for the interactions between a series of aromatic carboxylic acids and sarcosine anhydride are given in Table I. The apparent stability constants have been evaluated and are listed under the columns headed $K_1$ and $K_2$. The values listed under the heading $K_1$ were calculated on the basis of a one-to-one interaction while those listed under $K_2$ were determined on the assumption of an interaction involving two molecules of the acidic substance and one of sarcosine anhydride. Although the 2:1 stoichiometric ratio appears to be the one most frequently encountered in this study, it is difficult to use the apparent stability constant calculated on this basis as a sole measure of the complexing activity taking place in the system. The reason for this is twofold: the complex may be the result of a series of interactions and not necessarily the same in every case; and the compounds studied varied greatly in their original solubilities; and since this factor is squared in the calculation, the importance of these differences is exaggerated. The relative ability of these compounds to form complexes with sarcosine anhydride, however, can be qualitatively characterized by means of the constant calculated on the assumption of a 1:1 molecular ratio, or from the per cent increase in solubility with a 1% solution

TABLE VII.—INTERACTIONS OF VARIOUS AROMATIC POLAR COMPOUNDS WITH SARCOSINE ANHYDRIDE AT 25°

| Compound | Orig. Solubility Molar Concn. × 10² | $K_1$[a] (liter) (mole) | $K_2$[b] (liter) (mole) | Increase in Solubility With a 1% Sarcosine Anhydride Soln., % |
|---|---|---|---|---|
| Rutin | 0.0055 | 11.3 | $1 \times 10^6$ | 91 |
| Oxytetracycline | 0.04 | 2.5 | 2,600 | 17 |
| Melamine | 4.20 | 2.0 | 23 | 13 |
| Chloramphenicol | 1.21 | 1.7 | 68 | 12 |
| Quinine hydrochloride | 13.3 | 1.4 | 4.9 | 11 |
| Picric acid | 5.50 | 1.5 | 13 | 10 |
| Phenobarbital | 0.55 | 1.8 | 170 | 9 |
| L-Tyrosine | 0.31 | 1.4 | 220 | 7 |

[a] Apparent stability constant calculated on a 1:1 basis.
[b] Apparent stability constant calculated on a 2:1 basis.

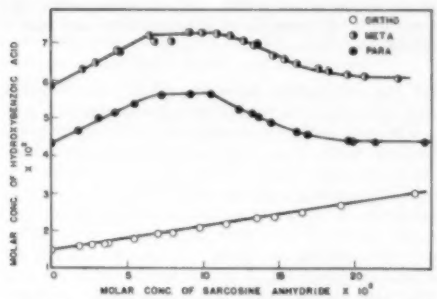


Fig. 4.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of the hydroxybenzoic acids in water at 25°. The system contained a total of 0.145 mole/L. of the *meta* and *para* isomers in and out of solution.

of the complexing agent. Although the solubility differences also influence the constants evaluated on a 1:1 basis, where the original solubilities are of the same magnitude, the effect is minor. Where a series of compounds show a wide variance in their original solubilities, the per cent increase in solubility seems to be the best method of comparing complexing activity.

It can be seen from Table I that for the hydroxybenzoic acids the *para* isomer exhibits the strongest complexing tendency, followed by the *ortho* and *meta* isomers. The phase diagrams describing these interactions are shown in Fig. 4. The apparent stability constants for the *para* and *meta* compounds were calculated on both sides of the plateau. When these constants were determined on the basis of a 1:1 ratio, the values obtained on the ascending and descending portions of the curve were not the same. If the stoichiometric ratio determined for the insoluble complex, 2:1, is utilized, however, the values for the constants do agree. This would seem to indicate a "sandwich" type structure in solution. The results for the interaction involving the aminobenzoic acids correspond to those for the hydroxybenzoic acids in that the order of activity is *para* > *ortho* > *meta*.

Since in an isomeric series of compounds there is a marked difference in the ability of the various substances to enter into complex formation, the position of the polar groups, as expected, appears to be important as well as the nature of these groups. The most stable complexes involving sarcosine anhydride would be expected where the polar molecule is capable of a two-point attachment with the nucleophilic centers of the complexing agent. The molecular models show that this condition is satisfied where the polar groups on a benzene ring are *para* to one another. The results obtained for the amino and hydroxybenzoic acids agree with this observation. The *ortho* isomers in both instances appear to form, however, more stable complexes than the *meta*.

It is interesting to note that in every instance, except for the *meta*-aminobenzoic acid, the increase in solubility with a 1% sarcosine anhydride solution was substantially greater than the maximum value of 8%, predicted from statistics for nonspecific interaction. This indicates a definite specific affinity of these compounds for sarcosine anhydride.

The binding tendency which aspirin exhibits for sarcosine anhydride was found to be of the same order as that shown by benzoic acid. This is to be expected if the reactions responsible for this tendency are between the electron-rich groups of the anhydride and the electrophilic groups of the acids. On this basis, however, the complexing activity of *ortho*-phthalic acid and *ortho* and *meta*-aminobenzoic acids should be greater than the observed results indicate. The only apparent explanation for these anomalies is an increase in the solute-solute and solute-solvent interactions with these substances.

The results obtained for the 4-aminosalicylic acid interaction gives an indication that the complexing activity of a multifunctional compound is, very crudely, the sum of the activities of each of its polar groups. If the "per cent increase" values listed in Table I are considered, it can be seen that *ortho*-hydroxybenzoic acid is increased in solubility by 30%, while benzoic acid only shows a 19% increase. Similarly *para*-aminobenzoic acid has a value 7% greater than that for benzoic acid. If the complexing properties were purely additive, the increase in solubility expected with 4-aminosalicylic acid would be 37%, which agrees very well with the value, 36% obtained experimentally.

The phase diagrams for the dihydroxybenzoic acids exhibit plateau regions indicating insoluble complex formation. The phase diagrams for the interactions of 2,4- and 3,4-dihydroxybenzoic acids with sarcosine anhydride are shown in Figs. 5 and 6, respectively. It was possible, in these cases, to calculate apparent stability constants for the reaction products. The other acids in this series, however, had no rising portion on the solubility curves, which made it impossible to calculate constants

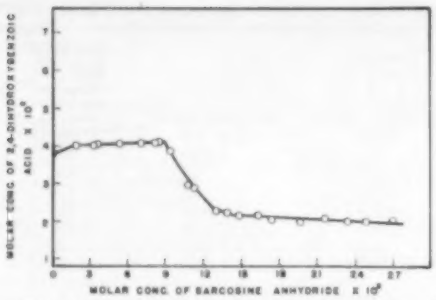


Fig. 5.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,4-dihydroxybenzoic acid in water at 25°. The system contained a total of 0.195 mole/L. of 2,4-dihydroxybenzoic acid in and out of solution.

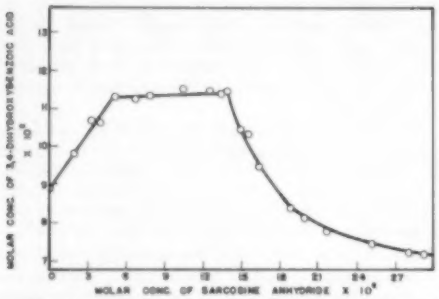


Fig. 6.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 3,4-dihydroxybenzoic acid in water at 25°. The system contained a total of 0.325 mole/L. of 3,4-dihydroxybenzoic acid in and out of solution.

for the interactions. When these constants were determined on the basis of a 2:1 molecular ratio, which is the stoichiometry of the insoluble complex, there was agreement between the values obtained from the ascending and descending portions of the curve. The only trihydroxybenzoic acid studied, gallic acid, also resulted in the formation of an insoluble complex and the phase diagram for this interaction is shown in Fig. 7. There was a small increase in the solubility of the acid in this case, which permitted the calculation of an apparent stability constant. The value of this constant, 64, determined on a 1:1 basis, indicates a strong complexing tendency of gallic acid for sarcosine anhydride. The constant calculated from the stoichiometry of the insoluble complex, 6:1, was $5.5 \times 10^4$ for the upcurve and $4.7 \times 10^4$ on the downcurve. A 6:1 interaction, however, would hardly seem likely in the solution phase.

Table II lists the aromatic acids which formed insoluble complexes with sarcosine anhydride along with the melting points and stoichiometric ratios of the complexes. A stoichiometry of two acid molecules to one molecule of sarcosine anhydride is found in the majority of insoluble complexes formed in this study. Gallic acid and 3,5-dihydroxybenzoic acid, however, form insoluble addition products which have six acid molecules associated with each molecule of complexing agent. The phase diagram

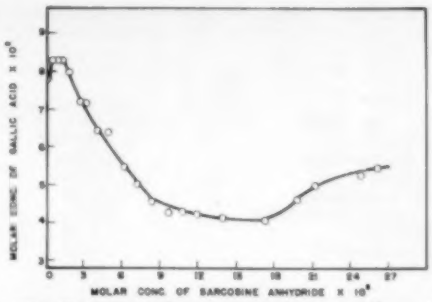


Fig. 7.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of gallic acid in water at 25°. The system contained a total of 0.147 mole/L. of gallic acid in and out of solution.

for the interaction between 3,5-dihydroxybenzoic acid and sarcosine anhydride is presented in Fig. 8.

The multihydroxybenzoic acids would be expected to form stable complexes with sarcosine anhydride since in every case, except 3,5-dihydroxybenzoic acid, the polar groups are situated so as to be conducive to a two-point attachment with the nucleophilic centers of the complexing agent. If the interacting molecules are joined at two points, the 2:1 complexes may result from one molecule of acid attaching to each side of the anhydride molecule to form a "sandwich." It is difficult, however, to rationalize higher stoichiometric ratios on this basis. Other reasonable explanations for these complexes may be that the acidic substances exist in solution as dimers, trimers, or polymers of the interacting species, or that a 1:1 reaction forms a nucleus which then associates with other molecules of the donor substance. A more detailed investigation of the insoluble materials including X-ray diffraction studies will be necessary to elucidate the nature of the interactions responsible for complexes of this type.

**Interactions of Sarcosine Anhydride With a Series of Aromatic Alcohols and Phenols.**—Table III lists the aromatic alcohols and phenols studied, along with some of their physical properties and the results of their interactions with sarcosine anhydride. The basis for choosing these compounds was to demonstrate better the effect of various polar groups and their positions on the binding tendencies of the parent molecule. Since these compounds vary so greatly in their original solubilities the per cent increase in solubility with a 1% sarcosine anhydride solution appears to be the best means of comparing their complexing tendencies.

Sarcosine anhydride and 8-hydroxyquinoline appear to interact significantly with a $K_1$ value of 8.2 as shown in Table III. The phase diagram for this interaction is illustrated in Fig. 9. There are two possible explanations to account for this relatively strong interaction: (*a*) the amphoteric nature of this compound would render the 8-hydroxyquinoline–sarcosine anhydride interaction quite favorable, and (*b*) the fused ring structure of the compound with its greater hydrophobic character would increase the influence of the "squeezing out" effect which has been discussed by Higuchi and Lach in a previous publication (4).

Gallacetophenone, phloroglucinol, and 2,5-dihy-

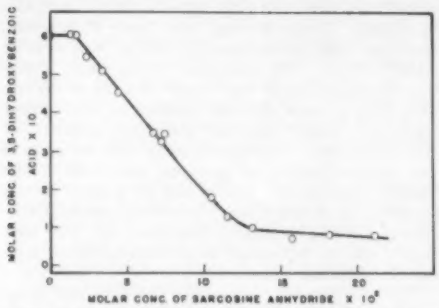


Fig. 8.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 3,5-dihydroxybenzoic acid in water at 25°. The system contained a total of 0.731 mole/L. of 3,5-dihydroxybenzoic acid in and out of solution.

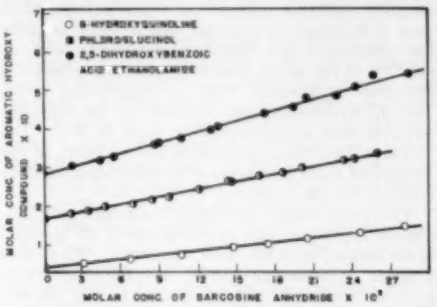
8-HYDROXYQUINOLINE
PHLOROGLUCINOL
2,5-DIHYDROXYBENZOIC ACID ETHANOLAMIDE
MOLAR CONC. OF AROMATIC HYDROXY COMPOUND X 10
MOLAR CONC. OF SARCOSINE ANHYDRIDE X 10

Fig. 9.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 8-hydroxyquinoline, phloroglucinol, and 2,5-dihydroxybenzoic acid ethanolamide in water at 25°.

droxybenzoic acid ethanolamide all showed relatively strong binding tendencies as would be expected with multihydroxy compounds. The phase diagrams for the interactions involving the latter two compounds are presented in Fig. 9. The comparatively strong complexing activity of 2,3-dihydroxyquinoxaline may be attributed to the highly dipolar nature of this fused ring compound.

The order of activity for the aminophenol isomers would be expected to be the same as that exhibited by the aminobenzoic acids. This was not the case, as the *ortho*-aminophenol showed greater activity than the *para* compound which in turn was more active than the *meta* isomer. It was interesting to note, that for this series of compounds the complexing activity and solubility seem to be related, i. e., the least soluble compound shows the greatest activity and the most soluble compound shows the least activity.

The relative binding tendencies of salicylamide, salicyl alcohol, and salicylamine were in the order expected. The molecular models show that a two-point attachment with the electron-rich centers of the complexing agent is possible with all three of these compounds. The N-hydrogen of the amide, however, would form this association more easily than the aliphatic alcohol or amine group. The relative inactivity of the salicylamine can be attributed to the ease with which intramolecular associations can occur in this molecule. The increase in solubility, 5% with a 1% sarcosine anhydride solution is also less than 8%, the maximum expected on a nonspecific, statistical basis, indicating the relatively weak affinity of this compound for the complexing agent. The slope of the solubility curve describing the interaction between salicyl alcohol and sarcosine anhydride is greater than one. Therefore, it appears that the reaction product has more than one molecule of the alcohol associated with each molecule of complexing agent; otherwise the results indicate that more sarcosine anhydride is present in the form of the complex than was added to the system.

The stoichiometric ratios and melting points of the insoluble complexes formed between sarcosine anhydride and the aromatic hydroxy compounds are listed in Table IV. It can be seen from the phase diagrams illustrated in Figs. 10 and 11 that the 1,5- and 2,7-naphthalenediol isomers both formed

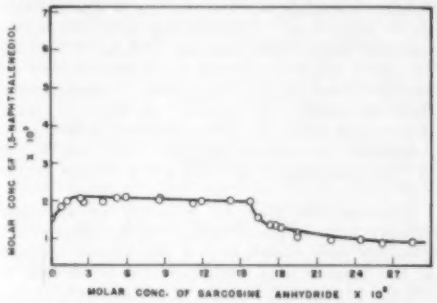


Fig. 10.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 1,5-naphthalenediol in water at 25°. The system contained 0.156 mole/L. of 1,5-naphthalenediol in and out of solution.

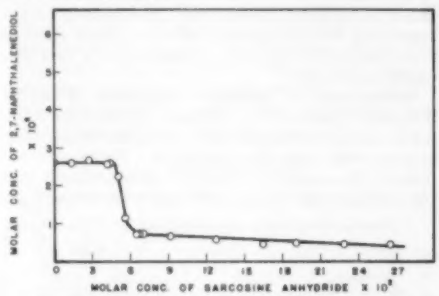


Fig. 11.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of 2,7-naphthalenediol in water at 25°. The system contained 0.156 mole/L. of 2,7-naphthalenediol in and out of solution.

insoluble reaction products. In the case of the 1,5 compound there was an initial rise in the solubility curve which permitted the calculation of an apparent stability constant. The stoichiometric ratio of the insoluble material was 1:1, consequently, the constant was determined on this basis. The values obtained from the portions of the curve with a positive and negative slope were in agreement indicating that the principal reaction occurring is of a 1:1 nature. The value of the constant, 23, is indicative of a strong binding tendency. A stable

complex would be expected with this compound in view of its fused ring system which would increase the influence of the "squeezing out" effect and the position of the polar groups which make possible a two-point attachment with the complexing agent. The 2,7-naphthalenediol resulted in an insoluble complex with a stoichiometric ratio of 3:1. The absence of an initial increase in the solubility of the compound made it impossible to calculate a stability constant for the complex.

The interactions involving hydroquinone, 2,6-dihydroxytoluene and hexylresorcinol all resulted in the formation of insoluble complexes. The phase diagram representing the hydroquinone-sarcosine anhydride interaction is illustrated in Fig. 12 and shows that the insoluble complex is precipitated immediately. A similar solubility curve was obtained for the 2,6-dihydroxytoluene and sarcosine anhydride interaction; therefore, the apparent stability constants of these reaction products could not be determined. A phase diagram for the hexylresorcinol interaction could not be obtained because the excess hexylresorcinol in the system formed an emulsion which made it impossible to obtain a sample of the solution for analysis. By using an excess of sarcosine anhydride it was possible, however, to precipitate an insoluble complex which was found by chemical analysis to have a 5:1 stoichiometric ratio.

Hydroquinone may be expected to associate strongly with sarcosine anhydride because of the favorable *para* relationship of the hydroxy groups on the benzene ring. Whether this is the case is difficult to establish because of the extreme insolubility of the complex, evidenced by the steep slope of the descending portion of the phase diagram and lack of any detectable initial increase in solubility with this compound.

The variety of stoichiometric ratios exhibited in this study may be rationalized on the basis discussed previously for the insoluble products which resulted from interactions between aromatic acids and sarcosine anhydride.

**Interactions of Sarcosine Anhydride With the Xylylene Glycols.**—The xylylene glycols were studied to determine the possible steric effects on interactions with sarcosine anhydride. From observations of the molecular models it would appear that the configuration of the *ortho* and *meta* isomers are suited for a two-point attachment with the complexing agent. It can be seen from Table V, however, that the order of complexing activity was *meta* > *ortho* > *para*. The reactivity of the *meta* isomer may be magnified in this study because of the use of a 20% sodium chloride solution as the solvent which was used to decrease the initial solubility of this compound. This would mean that the ratio of the volume fraction of sarcosine anhydride to the volume fraction of water would be greater in this system than in the other. Therefore, the relatively strong binding tendency exhibited by the *meta* isomer may be influenced by an increase in the nonspecific solvation effect.

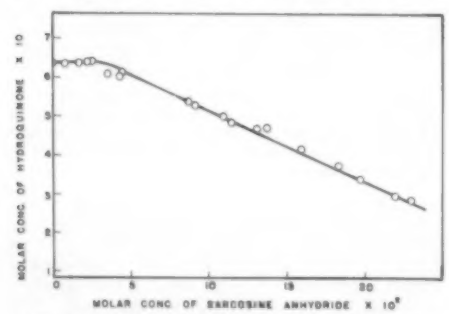


Fig. 12.—Phase diagram showing the effect of sarcosine anhydride on the apparent solubility of hydroquinone in water at 25°. The system contained 0.727 mole/L. of hydroquinone in and out of solution.

The solubility curves for both the *ortho*- and *meta*-xylylene glycols have slopes greater than one, indicating a stoichiometric ratio higher than 1:1 for these complexes. The *para* isomer showed a low degree of activity as would be expected for a compound of this type where the steric requirements were unfavorable and the polar groups are removed from the aromatic nucleus.

**Interactions of Sarcosine Anhydride With Aliphatic Dicarboxylic Acids.**—A series of aliphatic dicarboxylic acids were included in the study in order to show the influence of an aromatic structure on interactions of this type. Table VI lists the acids used in this study along with the results of the interactions and some physical properties of these aliphatic compounds. The solubility curves for these compounds demonstrate the weak interactions between sarcosine anhydride and nonaromatic acids. Since the carboxy groups of pimelic and suberic acids are at the proper distance for a two-point attachment with the sarcosine anhydride molecule, it appears, from the weak binding tendencies exhibited by these compounds, that the presence of an aromatic structure is essential for the donor substance to show a strong attraction for the complexing agent, sarcosine anhydride.

It is interesting to note that the complexing tendencies of these acids appear to be related to the chain length of the compounds in that the longer the carbon chain, the greater the tendency to complex. This may be due to the increased hydrophobic portion of the longer chained molecules and its resultant influence on the "squeezing out" effect.

The wide differences in the water solubilities of these substances may also play an important role in the relative reactivities of these aliphatic acids. For example, the high water solubility of pimelic acid would indicate a strong solute-solvent interaction which in turn would decrease the tendency of this compound to form a complex with sarcosine anhydride.

**Interactions of Sarcosine Anhydride With Various Other Polar Compounds.**—A group of miscellaneous compounds which were included in this study are listed in Table VII along with the results of their interactions with sarcosine anhydride and some of their physical properties.

Solutions of sarcosine anhydride have been used to solubilize rutin and similar compounds in several commercial preparations in order to make concentrated solutions of these substances to be used as injectables. The results of this study indicate that the solubilization of these substances may be the result of molecular complex formation.

The very strong complexing activity exhibited by

rutin is not unexpected when the aromatic character and abundance of phenolic hydroxy groups on the molecule are considered. These same factors are important in the interaction of oxytetracycline and sarcosine anhydride. The large difference in complexing activity between rutin and oxytetracycline can be attributed to the ease with which intramolecular hydrogen bonds can form in the oxytetracycline molecule.

Melamine gave results which were somewhat higher than expected in view of the fact that the molecule has no carboxylic acid or hydroxy group. These groups appear to have the greatest effect on the complexing tendencies of the compounds studied.

The binding tendency exhibited by chloramphenicol fits in nicely with the results obtained with the xylylene glycols. In both cases the degree of complexing can be considered to be influenced by the alcohol groups and aromatic nucleus. The quinine hydrochloride molecule also resembles these previously mentioned alcohols, differing in that it has only one hydroxy group but a larger aromatic nucleus and greater hydrophobic character. Thus, the degree of complexing activity exhibited by quinine hydrochloride is consistent with the results obtained for compounds with similar structures.

The relatively weak interactions between picric acid, phenobarbital, and L-tyrosine with sarcosine anhydride were not entirely unexpected. In the interaction involving picric acid, the presence of the nitro groups on the molecule may interfere with the ability on the phenolic hydroxy to associate with the complexing agent. While in the case of the barbituric acid derivative, none of the polar groups which appear to be necessary for strong binding with sarcosine anhydride are present. The inactivity of L-tyrosine can be ascribed to the intra- and intermolecular associations which are prevalent in this compound.

## DISCUSSION

The results of this investigation give definite evidence for the formation of both soluble and insoluble molecular complexes in aqueous solution between sarcosine anhydride and various proton-donor compounds. There is also an indication that the nature of the electrophilic groups and their position on the molecule influence the degree of complexation. Furthermore, the weak binding tendency encountered with the aliphatic dicarboxylic acids point out the importance of an aromatic system in these interactions. For a molecule to show optimum complexing activity with sarcosine anhydride, it appears that it should have an aromatic nucleus, one or more acid or hydroxy groups attached to the aromatic structure, and the electrophilic groups should be in the optimum position to make a two-point attachment with the nucleophilic centers of the complexing agent.

A comparison of the complexing tendencies of the acidic compounds indicates that the compounds with greater hydrophobic character show greater complexing activity than the more soluble substances. This correlation can be attributed to the increased influence of the "squeezing out" effect with the less soluble materials and the stronger solute-solvent interactions encountered with the more water-soluble donor compounds. Even with comparatively soluble compounds, however, relatively stable complexes can be formed if the steric relationships are favorable.

The stability constants for the complexes formed with sarcosine anhydride cannot be compared with the constants for the xanthine complexes, since with the anhydride there is evidence to indicate that interactions other than the 1:1 type are occurring simultaneously. The stability constants obtained by Higuchi and Kostenbauder (10), however, for the complexes formed between a series of water-soluble amides such as the N, N, N′, N′-tetramethyl derivatives of phthalamide, isophthalamide, terephthalamide, fumaramide, and succinamide; and chloramphenicol, *para*-hydroxybenzoic acid, and salicylic acid are of the same magnitude as the constants obtained in this study with the same donor compounds. It would appear reasonable, therefore, to expect the same type interactions to be responsible for the complexes with the substituted cyclic amide, sarcosine anhydride, as in the case of the previously mentioned water-soluble amides.

These findings lend further credence to the belief that interactions of this nature are important in mediating many enzymatic and drug actions. Since the diketopiperazine structure with its cyclic dipeptide linkage grossly resembles a segment of a protein alpha helix, the type of binding experienced for this compound is probably representative of those utilized by the biochemical macromolecules. The significant tendency of simple oxide groupings to form molecular complexes with a large array of organic compounds as shown in these studies, it is apparent, cannot be ignored in any detailed formulation of mechanism of *in vivo* actions.

## REFERENCES

(1) Higuchi, T., and Zuck, D. A., THIS JOURNAL, **41**, 10 (1953).
(2) *Ibid.*, **42**, 132(1953).
(3) *Ibid.*, **42**, 138(1953).
(4) Higuchi, T., and Lach, J. L., *ibid.*, **43**, 465(1954).
(5) *Ibid.*, **43**, 524(1954).
(6) *Ibid.*, **43**, 349(1954).
(7) *Ibid.*, **43**, 527(1954).
(8) Higuchi, T., and Kuramoto, R., *ibid.*, **43**, 393(1954).
(9) *Ibid.*, **43**, 398(1954).
(10) Higuchi, T., and Kostenbauder, H. B., *ibid.*, **45**, 518 (1956).
(11) Bilek, L., *et al.*, *Monatsh. Chem.*, **84**, 738(1953).
(12) Mylius, F., *Ber.*, **17**, 287(1884).

# Further Studies on the Effects of Ephedrine in the Presence of Cocaine*

By PHILLIP V. HAMMOND

**It has been demonstrated that cocaine will lessen some of the effects of ephedrine, chiefly cardiac output and blood pressure changes. The nature of the finer mechanism of action of these changes has not been ascertained. Because of the many unrelated sites of action it is believed there may possibly be more than one type of mechanism of action involved. Diminution of response to cocaine is largely cardiac in nature because vasoconstriction and increase in peripheral resistance in the femoral vascular bed was not marked. This action is chiefly central in origin because it cannot be demonstrated on the isolated rabbit heart. This indicates that the theory of Gaddum and Kwiatkowski (9) that the action of ephedrine is mediated chiefly through the presence of epinephrine cannot be demonstrated here, since cocaine will potentiate the action of epinephrine on the isolated rabbit heart. It is believed that the nature of the antagonism between ephedrine and cocaine is noncompetitive, e. g., the two drugs combine with different parts of the receptor mechanism.**

EPHEDRINE, an important and useful drug, bears many striking pharmacological resemblances to epinephrine; however, there exist several significant differences, such as the action of the two drugs *in vivo* in the presence of cocaine. We have found, as Tainter (1), Holck (2), and other investigators (3), that some of the effects of ephedrine in the cocainized animal are either greatly reduced or abolished.

Besides being of academic interest, it was felt that a further investigation of this phenomenon might cast additional light on the somewhat nebulous mechanism of action of ephedrine, and possibly elucidate the site or sites of action. With this in mind, it is the purpose of this paper to study the relationship of ephedrine to cocaine with special reference to the cardiovascular system.

## EXPERIMENTAL

**Pressor Response.**—Twenty-four mongrel dogs were used throughout the pressor and related experiments.

In conducting these experiments, an acute mammalian set-up was utilized. The procedure was to ascertain the normal blood pressure after the animal had become stabilized under pentobarbital sodium anesthesia (30 mg./Kg.). Ephedrine 0.3 mg./Kg. was administered intravenously, this was followed by cocaine administered subcutaneously in varying doses ranging from 4 mg./Kg. to 30 mg./Kg. After the animal had become cocainized (twelve to fifteen minutes) a second dose of ephedrine was given. This procedure was repeated three or four times in each experiment. In some of the experiments the animal was cocainized without a previous injection of ephedrine, and after the proper time interval ephedrine was administered. Pressure responses to ephedrine were also obtained from the femoral artery.

By setting up experiments in which ephedrine was administered at varying time intervals in the absence of cocaine it was determined that tachyphylaxis was not a factor in the diminution in response to the pressor action of ephedrine. Tachyphylaxis was ruled out when, after a suitable time interval, an injection of ephedrine produced the same pressor response as the original dose. Electrocardiographic changes were taken with a Sanborn Viso-Cardiette using standard limb leads.

**Isolated Heart.**—The action of ephedrine in the presence of cocaine was determined on the isolated rabbit heart using the Langendorff and Anderson (4) perfusion pump. The animal was sacrificed. The heart was quickly removed and placed into the heart chamber. Here it was perfused with an oxygenated, modified Ringer's solution at a constant temperature of 37.5°. After the steady amplitude and the steady rate of contraction had been determined, the two drugs were perfused through the preparation at definite intervals. Cocaine was used in doses ranging from 0.12 mg. to 0.25 mg. and ephedrine was used in doses ranging from 0.1 mg. to 1.32 mg.

**Action on Vascular Bed.**—The action of ephedrine on the vascular bed in the cocainized animal was tested by anesthetizing dogs in the manner previously mentioned. Five hundred U. S. P. units of heparin or 250 mg./cc. of mepesulfate[1] were administered. Throughout these experiments heparin, mepesulfate, or pontamine Fast Pink BL standard[2] were given when necessary as anticoagulants. Both femoral arteries were isolated and cannulated. Blood pressure readings were taken from the right femoral artery, whereas the arterial flow into the left vascular bed was measured from the left femoral artery by means of a Shipley and Wilson (5) rotameter. Changes produced were visual. Desirable changes were recorded by means of a

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Preliminary reports on certain types of the experiments herein described appeared in THIS JOURNAL, 47, 67(1958).

[1] Hoffmann LaRoche—Formerly known as Treburon.

[2] E. I. du Pont de Nemours and Co., Inc.

Sanborn Viso-Cardiette Model 572 M. A 500-cc. polyethylene bottle was used in the system as a dampening chamber. At the end of the experiments the rotameter was calibrated using a graduated cylinder and stop watch. In one experiment an eight-channel recorder was used to record aortic pressure, volume, and flow. Cocaine in doses ranging from 18–22 mg./Kg. was administered subcutaneously, whereas ephedrine in doses of 0.3 mg./Kg. was administered into the system by injecting it into a plastic chamber attached to a magnetic stirrer. A total of six dogs was used.

**Visceral Blood Vessels.**—The action of the two drugs was tested on visceral blood vessels by means of an acute mammalian set-up. The carotid artery was isolated and cannulated. The femoral vein was isolated and used as a site for the administration of ephedrine, whereas cocaine was administered subcutaneously. A portion of the small intestine was exposed and placed in an oncometer. Readings were made by means of kymograph.

**Cardiac Output.**—Cardiac output studies were continued by means of the radioactive isotope dilution method described by MacIntyre (6) and others, using iodinated ($I^{121}$) human serum albumin. Prior to the anesthetization of the dog, Lugol's solution was administered orally in order to prevent the thyroid from picking up the radioactive iodine. Pentobarbital sodium was used as the anesthetic agent. The jugular vein and femoral artery were isolated, and connulas and Cournand needles were inserted. Iodinated ($I^{131}$) human serum albumin was employed in doses of 30 to 100 $\mu$c. The dilution technique employed was to dilute 1 cc. of radioactive iodinated human serum albumin to 5 cc. with normal saline. Of this dilution, 0.1 cc. was diluted to 10 cc. One cubic centimeter of the aliquot was used as a control. The volume of the dose was 4.4 cc. The dilution curve was obtained by plotting the radioactivity of the blood flowing over the detector as a function of time.

**Oxygen Uptake.**—In an initial attempt to determine the mechanism of action of ephedrine in the cocainized animal, oxygen uptake studies employing the Warburg respirometer were undertaken. Male mice weighing 16–18 Gm. (Swiss strain) were used in these experiments. Cocaine was administered to the intact animal in doses of 40 mg./Kg. and ephedrine in doses of 2 mg./Kg. (Controls were administered approximately the same volume of saline as the treated mice.) After sacrificing the animals, the livers were rapidly removed and placed on ice. One hundred milligrams of sample per cc. of homogenate was used. The liver samples were homogenized in Tyrode solution to which 1% of glucose had been added. The tissues were then placed in Warburg flasks which were placed in a constant temperature bath of 37°. The flasks were equilibrated for ten minutes. Uptake of oxygen was measured in ten-minute periods for two hours. One dozen animals were used in these experiments.

## RESULTS AND DISCUSSION

**Pressor Response.**—Throughout all of the pressor experiments the effects of ephedrine on the blood pressure of the cocainized animal were greatly reduced. As seen in Fig. 1, the diminution in response

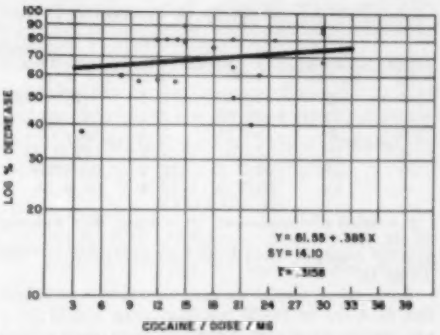


Fig. 1.—Diminution of pressor response to ephedrine in the cocainized animal.

to ephedrine was proportional to the dose of cocaine employed. For example, doses of 30 mg./Kg. of cocaine prevented 90% of the pressor response to ephedrine, whereas a dose of 10 mg./Kg. prevented 57% of the pressor response to ephedrine.

The response to ephedrine was found to be reduced following the administration of cocaine, when compared to the effects of the same dose of ephedrine given either before the injection of cocaine or after the effects of cocaine had worn off.

From the results obtained, the equation for the line was found to be $Y = 61.55 + 0.385X$. The standard error of estimate $Xy = 14.10$ and the correlation of index $Y = .3158$. Although the rho obtained may be indicative of an immoderate degree of correlation, it should be recognized that such experiments as those herein described often include biological functions which cannot be rigidly controlled and their combined influence on one or the other of the variables is difficult to determine. It is of importance, however, to note that the rho is such that it shows a trend toward a positive association between these variables.

In conducting the studies on tachyphylaxis and electrocardiographic changes the results obtained confirmed those findings previously reported by this laboratory (7), e. g., tachyphylaxis may be ruled out if sixty-five to seventy minutes are permitted between injections of ephedrine, and, whereas these drugs alone and combined produced the same significant electrocardiographic changes as previously reported, we could not make any definite conclusions from these changes alone.

**Isolated Heart.**—The results of 12 experiments revealed that the diminution in response to ephedrine cannot be satisfactorily demonstrated on the isolated rabbit heart. Both drugs increased the amplitude and rate of contraction until toxic levels were reached. In the nontoxic doses employed, the response to ephedrine was not lessened by the previous administration of cocaine.

**Vascular Bed.**—In Table I, both ephedrine and cocaine singularly produced vasoconstriction in the femoral vascular bed and an increase in peripheral resistance. The overall action of ephedrine in the presence of cocaine on the femoral vascular bed is largely additive rather than antagonistic to cocaine.

**Visceral Blood Vessels.**—The results of the testing of the two drugs on the visceral blood vessels, e. g., a portion of the small intestine, revealed the

TABLE I.—EFFECTS OF EPHEDRINE-COCAINE ON THE FEMORAL VASCULAR BED OF THE DOG

| Expt. No. | Dose-Cocaine, mg./Kg. | Normal P/F | R | Ephedrine, 2 mg./Kg. P/F | R | Cocaine RP/F | R | Ephedrine and Cocaine P/F | R |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 22 | 110/35 | 3.1 | 115/31.25 | 3.7 | 115/27 | 4.3 | 95/22.5 | 4.2 |
| 2 | 22 | 110/35 | 3.1 | 115/31.25 | 3.7 | 86/32.5 | 2.5 | 89/26.3 | 3.4 |
| 3 | 20 | 110/15 | 7.3 | 122/6.25 | 19.5 | 110/3.75 | 29.3 | 107/3.75 | 28.6 |
| 4 | 20 | 11/15 | 7.3 | 122/6.25 | 19.5 | 92/8.75 | 10.5 | 86/6.25 | 12.2 |
| 5 | 18 | 93/7.5 | 12.4 | 95/6.25 | 15.2 | 89/5 | 17.8 | 88/5 | 17.6 |
| 6 | 18 | 93/7.5 | 12.4 | 95/6.25 | 15.2 | 94/6.00 | 15.8 | 94/6.25 | 15.0 |

P = Mean arterial pressure. F = Flow. R = Resistance.

average changes taken from four experiments. See Table II.

From these results we may conclude that ephedrine in doses of 18–20 mg./Kg. does not produce any significant changes on the visceral blood vessels of the intestines.

**Cardiac Output.**—In Table III ephedrine produced a substantial increase in cardiac output. This effect was lessened by the action of cocaine. No adequate explanation is being offered to explain the variation in total peripheral resistance. Perhaps some of these variations were due to a compensatory action of the body. This increase in cardiac output, which was later modified by cocaine, was due mainly to an increase in stroke volume.

**Oxygen Uptake Studies.**—As a means of determining if oxygen utilization was involved in the mechanism of action of ephedrine in the cocainized animal, studies on oxygen uptake were conducted on the Warburg respirometer. In Fig. 2 these studies revealed that during the first hour liver homogenates from animals that had been previously treated with ephedrine gave off oxygen up to 18.63%; and that liver homogenates from animals that had been treated with cocaine took up a small amount of oxygen (a maximum of 8.27%), whereas liver homogenated from animals that had been treated with ephedrine and cocaine took up a maximum of 33.08% of oxygen during the second hour. It is believed that oxygen utilization will not explain the mechanism of action of ephedrine in the cocainized animals and that other enzymatic systems should be investigated. According to Axelrod (8) ephedrine is demethylated by an enzyme system in rabbit liver microsomes to norephedrine and formaldehyde. It is believed by this author that the giving-off of oxygen during the first hour might possibly be due to formaldehyde poisoning of an enzymatic system and that this action is overcome in time, e. g., within two hours, by the presence of norephedrine, enabling the liver homogenates to take up increasing amounts of oxygen and that this reaction is not affected by the presence of cocaine. This is a theory that needs further investigation, for at present there is not satisfactory scientific evidence to support it.

TABLE II.—ACTION OF EPHEDRINE-COCAINE ON VISCERAL BLOOD VESSELS

| | Normal | Ephedrine, 0.3 mg./Kg. | Cocaine 20 mg./Kg. | Ephedrine and Cocaine |
|---|---|---|---|---|
| Blood pressure, mm./Hg | 136 | 170 | 120 | 122 |
| Ht. of contraction of intestines, mm. | 10.8 | 10.6 | 10.6 | 10.6 |

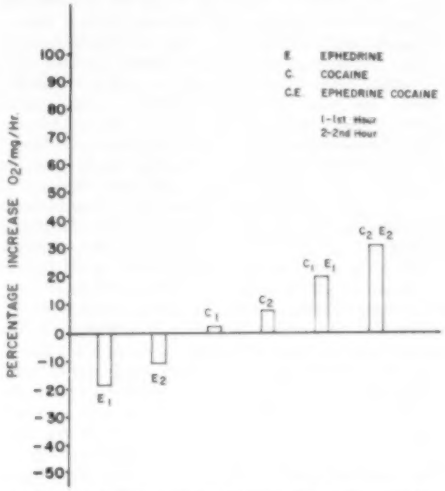


Fig.2.—Oxygen uptake, mouse liver homogenate.

## SUMMARY

1. The pressor response to a dose of 0.38 mg./Kg. is markedly reduced in the presence of cocaine. Under properly controlled conditions, this reduction amounts to 90 per cent or more.

TABLE III.—CARDIAC OUTPUT

| | Normal H1 | Normal H2 | Ephedrine[a] H1 | Ephedrine[a] H2 | Ephedrine[a] in the Cocainized Animal H1 | Ephedrine[a] in the Cocainized Animal H2 |
|---|---|---|---|---|---|---|
| Cardiac output, l./min. | 3.63 | 1.7 | 5.77 | 2.4 | 5.33 | 1.5 |
| Cardiac index, l./min./M² | 4.5 | 3.5 | 7.2 | 5.2 | 6.67 | 3.3 |
| Total peripheral resistance, dynes/cm.² | 2,564 | 1,328 | 1,605 | 1,328 | 1,738 | 3,444 |
| Stroke volume, cc./beat | 21.4 | 10 | 32 | 15 | 30.5 | 9 |

[a] 0.3 mg./Kg.

Diminution in response to the pressor effects of ephedrine in the cocainized animal is not related to tachyphylaxis.

2. The mechanism of action of the reduction to ephedrine cannot be explained on the basis of differences in electrical potentials taken from electrocardiographic changes.

3. The reduction in response to ephedrine in the presence of cocaine cannot be demonstrated on the isolated rabbit heart.

4. Both ephedrine and cocaine singularly acted on the femoral vascular bed to produce some degree of vasoconstriction and a decrease in blood flow. However, in the presence of cocaine, the vasoconstriction and decrease in blood flow is augmented rather than decreased.

5. Ephedrine and cocaine do not produce any significant action on visceral blood vessels.

6. The increase in cardiac output induced by ephedrine is greatly lessened by cocaine.

7. Differences in oxygen uptake of ephedrine and ephedrine in the presence of cocaine in liver homogenates could not satisfactorily explain the diminution in the cardiac output induced by cocaine.

## REFERENCES

(1) Tainter, M. L., *J. Pharmacol. Exptl. Therap.*, **36**, 569 (1929).
(2) Holck, H. G. O., *Proc. Soc. Exptl. Biol. Med.*, **30**, 1087 (1933).
(3) Shinyo, I., *Folia Pharmacol. Japon.*, **49**, 210(1953).
(4) Anderson, F. F., and Crover, B. N., *J. Pharmacol. Exptl. Therap.*, **93**, 135(1948).
(5) Shipley, R. E., and Wilson, C., *Proc. Soc. Exptl. Biol. Med.*, **78**, 724(1951).
(6) MacIntyre, W. J., Pritchard, W. H., Eckstein, R. W., and Fridell, H. L., *Circulation*, **4**, 552(1951).
(7) Hammond, P. V., THIS JOURNAL, **47**, 67(1958).
(8) Axelrod, J., *J. Pharmacol. Exptl. Therap.*, **114**, 430 (1955).
(9) Gaddum, J. H., and Kwiatkowski, J., *J. Physiol.*, **94**, 87 (1938).

# Drug Modification of Runway Behavior of Mice Influenced by Aversive Stimulus*

By J. A. HUNTZINGER†, J. J. WITOSLAWSKI, and H. M. HANSON

**Chlorpromazine administered intraperitoneally to white mice significantly increases running time on a short runway; this effect is proportional within the range studied with increasing dosage. Following exposure to an aversive stimulus, untreated mice or mice receiving the 2 mg./Kg. of chlorpromazine markedly increased running times as a result of such stimulation. However, mice receiving the 4 mg./Kg. dose showed no increase in running time. Assuming that such increases in running time are free of interaction with the preceding level of performance and are comparable in terms of absolute differences between groups it would appear that the compound either prevented the setting up of some condition ("fear") resulting from the light stimulus, which increased the running time in the untreated mice, or it prevented the operation of the condition set up by such aversive stimulation.**

THIS STUDY investigated the possibility that one of the traditional techniques for the study of instrumental behavior might be useful as a rapid first evaluation of unknown compounds believed to have psychotropic activity. A precursor of such an application was Brady's study of the effects of tetraethylammonium on the runway behavior of rats (1). It was felt that by a modification of the basic method, a response to so-called "fear"-producing stimuli might become measurable. Chlorpromazine hydrochloride, 10-(γ-dimethylaminopropyl) - 2 - chlorophenothiazine hydrochloride, was selected for testing as a reference agent for drugs of the "tranquilizer" class.

## METHOD

Eighty male albino mice (Merck Sharp & Dohme strain) sustained on a twenty-two-hour food deprivation schedule were trained to run a 24-inch elevated runway.

A timing zone of 17 inches was marked off on the runway equidistant from each end, the remaining space being designated starting and goal areas. The mice were reinforced by being allowed to eat for approximately three seconds from a supply of wet mash placed at the far end of the goal area. After a day's experimental session, the mice were fed wet mash *ad libitum* for one hour, water always being available.

After 25 training trials, the mice were randomly divided into four groups. On the next experimental day, after two trial runs, chlorpromazine was administered intraperitoneally to each group at the following dose levels: Control (saline), 2 mg./Kg., 4 mg./Kg., and 6 mg./Kg. Forty-five minutes postinjection, 4 trials were run. Running times were measured on the last preinjection trial and on the second and fourth postinjection trials. The time required to complete a run between the two 17-inch markers was recorded in tenths of a second. At the end of the third run while the mouse was still in the goal area and consuming the mash, a photoflood lamp placed approximately six inches above and in front of the mouse was

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illuminated for one second. This stimulus produced freezing and/or withdrawal in all mice. On the basis of earlier studies with rats it can be considered aversive, i. e., it would probably be sufficient to support instrumental escape behavior (2, 3). The mouse was then immediately returned to the starting area and the fourth and final trial given. If the mouse had not completed the run in sixty seconds, the trial was terminated by removing the mouse from the runway, a score of sixty seconds being recorded.

## RESULTS

The results are shown in Fig. 1. The running times measured in tenths of a second are plotted by group averages against the dose levels tested. The dashed line shows the mean preinjection running times for each group. There were no significant differences (tested by Kruskal-Wallis one-way analysis of variance (4)) at the 0.05 level for the mean preinjection running times for the 4 groups. Mean running times forty-five minutes after the injection of chlorpromazine are shown in the post-injection curve. An orderly increase in running times was obtained with increasing drug dosage. The differences between the group means were significantly different at the 0.001 level of confidence. However, differences between pre- and post-injection runs for the 0 and 2 mg./Kg. groups, tested separately by Mann-Whitney U. test, were not significantly different at the 0.05 level (4). The third curve in Fig. 1 labeled "poststimulus," shows the effect of the presentation of the brilliant light upon running times, and the interaction of this effect with the drug action. In the saline control group and the 2 mg./Kg. drug group the mean running times on the trial following the light stimulus were significantly different from the mean postinjection running times, at better than the 0.05 level of confidence. (This increase supports the notion that the light was aversive.) The 4 mg./Kg. dose did not significantly increase the mean running time when compared to the postinjection trial suggesting an interaction of the two variables: stimulus presentation and drug. The poststimulus curve is flatter than the postinjection curve, with intersection of the two curves near the 4 mg./Kg. dose value suggesting an inverse relationship with dose.

The poststimulus mean running time for the 6 mg./Kg. group indicates a *marked decrease* in running times following the presentation of the light stimulus and was found to be significantly different from the postinjection mean running time. However, there was a decrease in the size of this group (to N = 12) by the time of the stimulation trial due to the heavy sedation produced by the injected compound. Considering that probably only those animals most resistant to the effects of chlorpromazine were performing at the time of testing, it is possible that those animals might also be resistant to the effects of the stimulation. For these reasons this value is probably not germane to a discussion of the results of this study.

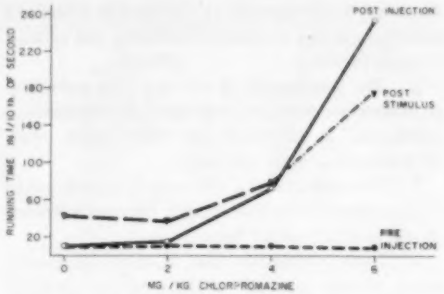



Fig. 1.—Average running times on an elevated runway for four groups of mice. Each point represents the average group performance on a single trial. Following a control trial (curve labeled "pre-injection") chlorpromazine was administered at the doses indicated on the abscissa. The effects of the drug are shown in the curve labeled "postinjection." The results of a final trial immediately preceded by exposure to an aversive stimulus are shown in the third curve. The 6 mg./Kg. point in this curve due to reduction in group size can only be considered a suggestive value.

## DISCUSSION

The particular experimental situation utilized for this study allows an approach to a condition more comparable to "emotion" in humans than most others generally in use in small animal experimentation. The response to repeated application of aversive stimulation in rodents rapidly changes with the replication of such stimulation. The first few times shock or other aversive stimulation is applied, gross effects considered as concomitants of "emotion" such as defecation, urination, pilo-erection, respiratory changes, hyperactivity, and/or "freezing behavior" are observed. After a number of applications of such stimulation most of these signs are completely absent, suggesting that as an "analogue" of "emotion" the response to chronic aversive stimulation is questionable. Conceivably by presenting the aversive stimulus only once and measuring the response to such stimulation immediately thereafter, as was done in the present study, a state closer to "true emotion" is made available for study.

## REFERENCES

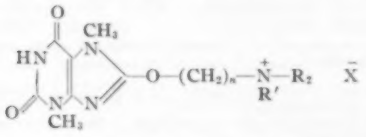
(1) Brady, J. V., *J. Comp. Physiol. Psychol.*, **46**, 307(1953).
(2) Kaplan, M., *ibid.*, **45**, 538(1952).
(3) Keller, F. S., *Psychol. Rec.*, **4**, 235(1941).
(4) Siegal, S., "Nonparametric Statistics for the Behavioral Sciences," McGraw-Hill Publishing Co., New York, N. Y., 1956.

# Quaternary Ammonium Salts of 8-(Dialkylaminoalkoxy)-theobromines and -caffeines as Curariform Agents*

By DIPTISH C. CHAKRAVARTY and JAMES W. JONES

**Three 8-(dialkylaminoalkoxy)-theobromine compounds were prepared by reacting the appropriate sodium dialkylamino alcoholate with 8-chlorotheobromine. Nine quaternary salts were prepared from the above three compounds by reacting each with methyl iodide, ethyl iodide, and benzyl chloride. The nine quaternary compounds along with nine corresponding caffeine quaternary compounds, previously reported, were tested for their curariform activity.**

CONTEMPORARY STUDIES with synthetic curariform compounds began with Bovet's working from a model of (+)-tubocurarine (1). This was followed by the synthesis and testing of a large number of quaternary ammonium compounds. Choline derivatives were among these (2). Included among the curariform agents studied by Bovet was tris-(β-triethylammonium-ethoxy)-1,2,3-benzene triiodide (Flaxedil), which was found to be most active (3). The present study was instituted with the view of preparing a group of compounds related to phenylcholine ethers and carrying out a very preliminary testing for possible curariform activity. Consequently a number of aminoalkyl ethers and their quaternary ammonium salts having the following general formula have been prepared from 8-chlorotheobromine.



The present syntheses represent an extension of the work with the corresponding derivatives of caffeine (4).

## EXPERIMENTAL

**8-Chlorotheobromine.**—This was prepared according to Biltz and Beck (5). Fifty grams of theobromine, suspended in 500 ml. of dry chloroform was refluxed at room temperature while a stream of dried chlorine was bubbled through it for six hours. The 8-chlorotheobromine was separated from the reaction mixture and recrystallized from glacial acetic acid. Yield—98%, m. p. 297°.

**8 - (Dialkylaminoalkoxy) - theobromines.**—The sodium amino alkoxides were prepared in a 500-ml., three-necked flask fitted for refluxing by allowing 2.6 Gm. (0.12 mole) of sodium metal, cut into small pieces, to react with 0.18 mole of the appropriate alcohol (dimethylaminoethanol, diethylaminoethanol, or diethylaminopropanol) in 50 ml. of dry benzene. The mixture was warmed over a water bath to complete the reaction as indicated by the absence of sodium particles. 8-Chlorotheobromine (0.12 mole) was added and refluxing with stirring was continued for two hours, by which time the reaction was complete. The solution was filtered while hot. The filtrate was transferred to a separatory funnel, washed three times with 50-ml. portions of water, dried over anhydrous sodium sulfate, and refiltered. Benzene was then removed under reduced pressure. Another 50-ml. portion of benzene was added and subsequently removed as above. The damp material was dried in an oven at 60° and recrystallized from benzene solution. The melting points and analyses are shown in Table I.

**Quaternary Salts of 8-(Dialkylaminoalkoxy)-theobromines.**—The aminoalkyl ether (0.01 mole) was dissolved in 50 ml. of absolute ethanol, and 0.015 mole of methyl iodide, ethyl iodide, or benzyl chloride was added with thorough stirring. The solution was placed in a refrigerator until the quaternary salt crystallized out. The salt was filtered out while cold, redissolved in absolute ethanol, and precipitated with ether. This process was repeated for each salt until a constant melting point was obtained. The melting points, analyses, and yields of the quaternary salts are given in Table II.

**Pharmacological Studies.**—These studies were only preliminary in scope to determine whether or not the compounds exhibited sufficient curariform activity to justify a complete activity study. They were carried out by the method used primarily for testing the curariform activity. A modification of the screen-drop test described by Cavillito, *et al.* (6), and the head-drop test (7) were used to determine the relative potencies of the compounds. In the mouse screen-drop test, groups of twelve white mice were used at each dosage level. The $ED_{50}$ was determined using the method of probit analysis described by Burn (8). The agents were administered intraperitoneally in 0.25 ml. of solution.

TABLE I.—8-(DIALKYLAMINOALKOXY)-THEOBROMINES

| R | n | M. p. | Yield, % | —Nitrogen, %— Calcd. | Found |
|---|---|---|---|---|---|
| $CH_3$ | 2 | 149–150 | 26 | 26.2 | 26.1 |
| $CH_3CH_2$ | 2 | 172–173 | 25 | 23.7 | 23.7 |
| $CH_3CH_2$ | 3 | 161–162 | 23 | 22.6 | 22.6 |

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Abstract of a dissertation submitted by Diptish C. Chakravarty to the Graduate College, State University of Iowa, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacy.

TABLE II.—QUATERNARY SALTS OF 8-(DIALKYLAMINOALKOXY)-THEOBROMINES

| R | n | R′ | X | Yield, % | M. p. | Nitrogen, % Calcd. | Nitrogen, % Found |
|---|---|---|---|---|---|---|---|
| $CH_3$ | 2 | $CH_3$ | I | 23 | 87– 88 | 17.1 | 17.1 |
| | | $CH_3CH_2$ | I | 20 | 126–128 | 16.5 | 16.5 |
| | | $C_6H_5CH_2$ | Cl | 12 | 131–133 | 17.5 | 17.5 |
| $CH_3CH_2$ | 2 | $CH_3$ | I | 20 | 106–107 | 16.0 | 16.0 |
| | | $CH_3CH_2$ | I | 25 | 96– 98 | 15.5 | 15.5 |
| | | $C_6H_5CH_2$ | Cl | 10 | 111–112 | 16.6 | 16.6 |
| $CH_3CH_2$ | 3 | $CH_3$ | I | 25 | 56– 57 | 15.5 | 15.4 |
| | | $CH_3CH_2$ | I | 24 | 76– 78 | 15.0 | 15.1 |
| | | $C_6H_5CH_2$ | Cl | 13 | 102–103 | 16.1 | 16.1 |

In the head-drop tests, groups of two rabbits were used. The concentrations of the solutions were adjusted to give a head-drop within a volume of 1–2 ml. The list of the compounds tested along with the results are included in Table III. The series of quaternary salts previously synthesised by these authors (4) were included in these tests.

## DISCUSSION

The same procedure was satisfactory for preparing the methiodides, ethiodides, and benzchlorides of the dialkylaminoalkoxy derivatives of theobromine, which was not true for preparing the corresponding caffeine-substituted quaternaries (4). As indicated in Table III, the relative activities of the compounds differ in the mouse and the rabbit which may be ascribed to species variation, as previously reported (9). The theory that more than one quaternary ammonium group is essential for curare-like activity, as in (+)-tubocurarine, does not hold here. The distance-activity relationship theory, which has been criticized by later investigators, proposes that the distance between onium heads has a direct relationship to the attainment of maximum activity. This theory is also without support in this study, since the compounds have only one onium head. No conclusion is drawn as to why only one theobromine derivative exhibited activity while seven caffeine derivatives were active.

The degree of activity exhibited by the compounds did not warrant further pharmacological investigations.

TABLE III.—PARALYZING DOSES IN THE PRELIMINARY MOUSE SLOPING-SCREEN AND RABBIT HEAD-DROP TESTS

| R | n | R′ | X | Mouse Tests, Dose, 50 mg./Kg. | Rabbit Tests, mg./Kg. |
|---|---|---|---|---|---|
| Theobromine Derivatives | | | | | |
| $CH_3$ | 2 | $CH_3$ | I | 0[a] | 0 |
| | | $CH_3CH_2$ | I | 0 | 0 |
| | | $C_6H_5CH_2$ | Cl | 0 | 0 |
| $CH_3CH_2$ | 2 | $CH_3$ | I | 0 | 0 |
| | | $CH_3CH_2$ | I | 0 | 0 |
| | | $C_6H_5CH_2$ | Cl | 0 | 0 |
| $CH_3CH_2$ | 3 | $CH_3$ | I | 72.5 | 17.4 |
| | | $CH_3CH_2$ | I | 0 | 0 |
| | | $C_6H_5CH_2$ | Cl | 0 | 0 |
| Caffeine Derivatives | | | | | |
| $CH_3$ | 2 | $CH_3$ | I | 168.3 | 50.9 |
| | | $CH_3CH_2$ | I | 37.6 | 10.8 |
| | | $C_6H_5CH_2$ | Cl | 57.3 | 5.6 |
| $CH_3CH_2$ | 2 | $CH_3$ | I | 38.1 | 12.8 |
| | | $CH_3CH_2$ | I | 39.3 | 15.4 |
| | | $C_6H_5CH_2$ | Cl | 124.6 | 42.1 |
| $CH_3CH_2$ | 3 | $CH_3$ | I | 38.2 | 13.3 |
| | | $CH_3CH_2$ | I | 0 | 0 |
| | | $C_6H_5CH_2$ | Cl | 0 | 0 |
| Flaxedil | | .. | .. | 4.1 | 0.47 |

[a] Inactive.

## REFERENCES

(1) Bovet, D., Courvoiser, S., Ducrot, R., and Horclois, R., *Arch. intern. pharmacodynamie*, **80**, 137(1949).
(2) Burger, A., "Medicinal Chemistry," Vol. I. Interscience Publishers, Ltd., London, 1951.
(3) Bovet, D., Depierre, F., and de Lestrange, Y., *Compt. rend.*, **223**, 74(1947).
(4) Chakravarty, D. C., and Jones, J. W., THIS JOURNAL, **47**, 233(1958).
(5) Biltz and Beck, J. pr. (2) **118**, 155.
(6) Cavillito, C. J., Soria, A. E., and Hoppe, J. O., *J. Am. Chem. Soc.*, **72**, 2661(1950).
(7) Varney, R. F., Lingar, C. R., and Halday, H. A., *Federation Proc.*, **7**, 261(1948).
(8) Burn, J. H., "Biological Standardization," Oxford Univ. Press, London, 1950, p. 347.
(9) Randall, L. R., *Ann. N. Y. Acad. Sci.*, **84**, 460(1951).

# Book Notices

*Laboratory Guide in Pharmacology.* By HARALD G. O. HOLCK, TOM S. MIYA, NORRIS W. DUNHAM, and GEORGE K. W. YIM. Burgess Publishing Co., 426 South 6th St., Minneapolis 15, Minn., 1959. iv + 115 pp. 22 x 27.5 cm. Paperbound. Price $3.50.

The manual is designed as an adjunct to lecture and textbook material, and only appropriate background information precedes the experimental procedures instead of detailed discussions of the pharmacological principles involved. Well-known procedures for preparing animals or isolated organs are not described in full detail. The manual is intended to acquaint the beginning student with various pharmacological principles in general without complicated and costly equipment. Most of the experiments are planned for student participation, and the procedures have been kept as simple as possible.

*Pharmazeutisches Wörterbuch, Dritten Auflage.* By CURT HUNNIUS. Walter De Gruyter & Co., Genthiner Strasse 13, Berlin W 35, Germany, 1959. xi + 731 pp. 12.5 x 18.5 cm. Price DM 32.

The first edition of this dictionary of pharmaceutical terms was published in 1950. The third edition has been enlarged, and includes definitions of a substantial number of terms not covered in the second edition, which was published in 1954. The definitions appear to be quite adequate, and for medicinal chemicals included in the *Deutschen Arzneibuch 6*, structural formulas, methods of preparation, and certain physical and chemical properties are given. Trademark names of drugs in this class are also included. The value of the dictionary is enhanced by an appendix including 17 tables of information of pharmaceutical interest. For example, antidotes, incompatibilities, maximum doses, and the relationship between different biological units are presented in tabular form.

*Progress in Biochemistry.* By FELIX HAUROWITZ. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, N. Y., 1959. xii + 358 pp. 15 x 22.5 cm. Price $8.50.

This book is the fifth in a series of reports on the progress in biochemistry since 1914. In selecting material for this report, the author has given preference to the fundamental aspects of biochemistry rather than to those describing methods or applications.

*Les Médicaments du Système Nerveux Cérébro-spinal.* Edited by F. MERCIER. Masson et Cie., 120, boulevard Saint-Germain, Paris 6e, France, 1959. 573 pp. 16.5 x 24.5 cm. Paperbound. Price 5,800 fr.

This book (in French) covers the drugs affecting the central nervous system and includes discussions on related procedures such as electroencephalography and artificial hibernation. The text includes chapters on: general anesthesia, preanesthesia and sedation, hypnotics and neurosedatives, neuroleptics, analgesics, antiparkinsonism drugs, antiepileptic drugs, nervous stimulants, developed tolerance, toxicomania, neuropsychiatry, drugs acting on the neuromuscular junction, and local anesthetics. The book is intended to serve as a guide to students and practitioners. A good index (author and subject) is appended.

*Précis de Thérapeutique et de Pharmacologie.* 1959 Supplement to the 9th ed. (1950). By RENÉ HAZARD. Masson et Cie., 120, boulevard Saint-Germain, Paris 6e, France, 1959. 138 pp. 13.5 x 20 cm. Paperbound. Price 320 fr.

This supplement (in French) includes concise monographs on newer therapeutic agents. Generic, chemical, and trade names are given, but the manufacturers are not indicated.

*An Introduction to Chemical Engineering.* By CHARLES E. LITTLEJOHN and GEORGE F. MEENAGHAN. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. xiii + 271 pp. Price $7.80.

This book is intended primarily as a beginning text for students in chemical engineering and related professions. It presents material with which the student should become familiar before undertaking a study of unit operations and thermodynamics. It attempts to explain some of the fundamentals upon which chemical engineering theory is based.

*Source Book of Industrial Solvents.* Vol. III Monohydric Alcohols. By IBERT MELLAN. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. v + 276 pp. 15 x 23 cm. Price $10.

This book is the third in a series, and in the latest volume the physical properties, azeotropic mixtures, and uses of industrial alcohols are presented. Many significant tables and illustrations are included. Since alcohol solvents enter into nearly every kind of industrial activity, this book should enjoy wide acceptance for a convenient source of information on these substances.

*Yardsticks for Industrial Research.* By JAMES B. QUINN. The Ronald Press Co., 15 East 26th St., New York 10, N. Y., 1959. v + 224 pp. 15 x 23 cm. Price $6.50.

The material presented in this book is based mainly upon a survey of practices of successful companies in evaluating industrial research output for management purposes. Using this approach, the author has developed a practical evaluation system designed to supply guidance to management. By means of the segmental approach, criteria and procedures are provided for both the technical and economic assessment of research output. The book probably will appeal most to research workers and executives who administer research programs.

*Steroids.* By LOUIS F. FIESER and MARY FIESER. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1959. xvii + 945 pp. 15 x 23.5 cm. Price $18.

A completely revised account of the entire field of steroids from the viewpoint of modern theory is presented in this book. It has been designed to replace "Natural Products Related to Phenanthrene," third edition (1949). The material in each chapter has been organized into a series of related topics unifying the discussion of the early history and the ensuing developments. All literature has been covered to about February 1959. Steroids are of especial interest in the pharmaceutical field because so many medicinal chemicals fall within this classification. The book has been designed in a very competent manner, which will appeal both to experts and students whose main interests lie in the field of steroid chemistry.

*Elementary Statistics With Applications in Medicine and the Biological Sciences.* By FREDERICK E. CROXTON. Dover Publications, Inc., 180 Varick St., New York 14, N. Y., 1959. vii + 376 pp. 13.5 x 20.5 cm. Paperbound. Price $1.95.

Assuming only a modest knowledge of mathematics and no prior knowledge of statistics, this book is designed to show how statisticians arrive at their results, how to interpret statistics, and how to test their validity. Numerous examples are given from fields of investigation that are familiar to most research workers in the biological sciences.

*Translation from German for Chemists.* By H. H. NEVILLE and W. E. YUILL. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xi + 139 pp. 13.5 x 18 cm. Price $2.50.

The aim of this book is not to teach the chemist German, but to equip him with a technique for reading, translating, and understanding German chemical material. Only those features of the language which are necessary for an intelligent use of the dictionary are presented. No previous knowledge of German is required to use this book, but some background would make the subject matter easier to follow.

*Brief Course in Organic Chemistry.* 2nd ed. By LYELL C. BEHR, REYNOLD C. FUSON, and HAROLD R. SNYDER. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. viii + 289 pp. 15 x 23 cm. Price $5.75.

This revised and expanded edition of the text presents an introduction to organic chemistry and includes the application of organic compounds. The revision contains references to many substances of practical value such as vitamins, hormones, other natural products, medicinals, agricultural chemicals, and other substances of biological interest. New chapters on homologous series, naturally occurring esters, and sulfur compounds have been added, and there are new illustrative photographs and drawings of molecular structures. Aromatic and heterocyclic compounds are introduced early and the similarities among aliphatic, aromatic, and heterocyclic compounds, rather than their differences, are stressed.

*Textbook of Toxicology.* By KENNETH P. DU BOIS. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1959. x + 302 pp. 14 x 21.5 cm. Price $6.50.

This book was written to fill the need for a textbook on toxicology containing subject matter in a concise, understandable form. *"Textbook of Toxicology"* provides a concise but thorough coverage of factual information concerning the major classes of the mode of action of poisons discussed at the cellular level. The practical circumstances under which poisoning can occur, diagnosis of poison, and treatment are also adequately discussed. The text stresses the toxic effects of the classes of chemical compounds rather than attempting a complete coverage of all toxic chemical agents. Chapters on newer subjects such as ionizing radiations, household poisons, and pesticides are included as well as subjects of long-standing importance such as metals, air-borne poisons, solvents, and plant and animal poisons. The medicolegal aspects, history, and general principles of toxicology are also discussed. The book is designed for use by medical students, graduate students, and advanced undergraduates, but it will also be of great value as a quick reference work in emergencies.

*Anatomy and Physiology.* Vol. I. By EDWIN B. STEEN and ASHLEY MONTAGU. Barnes & Noble, 105 Fifth Ave., New York 3, N. Y., 1959. xv + 332 pp. 13.5 x 21 cm. Paperbound. Price $2.50.

This book, one of the College Outline Series, presents a survey covering cells, tissues, integument, skeletal, muscular and digestive systems, blood, lymph, and circulatory system. It is keyed to standard textbooks on anatomy and physiology.

*Carcinogenesis. Mechanisms of Action.* Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xii + 336 pp. 13.5 x 20.5 cm. Price $9.50.

This book includes a series of papers presented during the 1958 Ciba Foundation Symposium which was held in London. All of the papers presented were prepared by experts in the field of carcinogenesis.

*Regulation of Cell Metabolism.* Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xi + 387 pp. 13.5 x 20.5 cm. Price $9.50.

A series of papers presented to a 1958 symposium on the regulation of cell metabolism is included in this book. This new symposium from the Ciba Foundation draws upon the collective experience of 39 internationally known authorities to present the latest research findings in the regulation of cell metabolism. The book is profusely illustrated with halftones and charts which clarify and augment the textual discussions. This volume should be of special interest to everyone involved in basic cell research.

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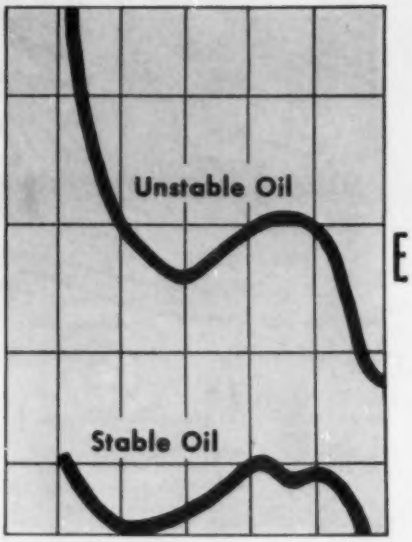


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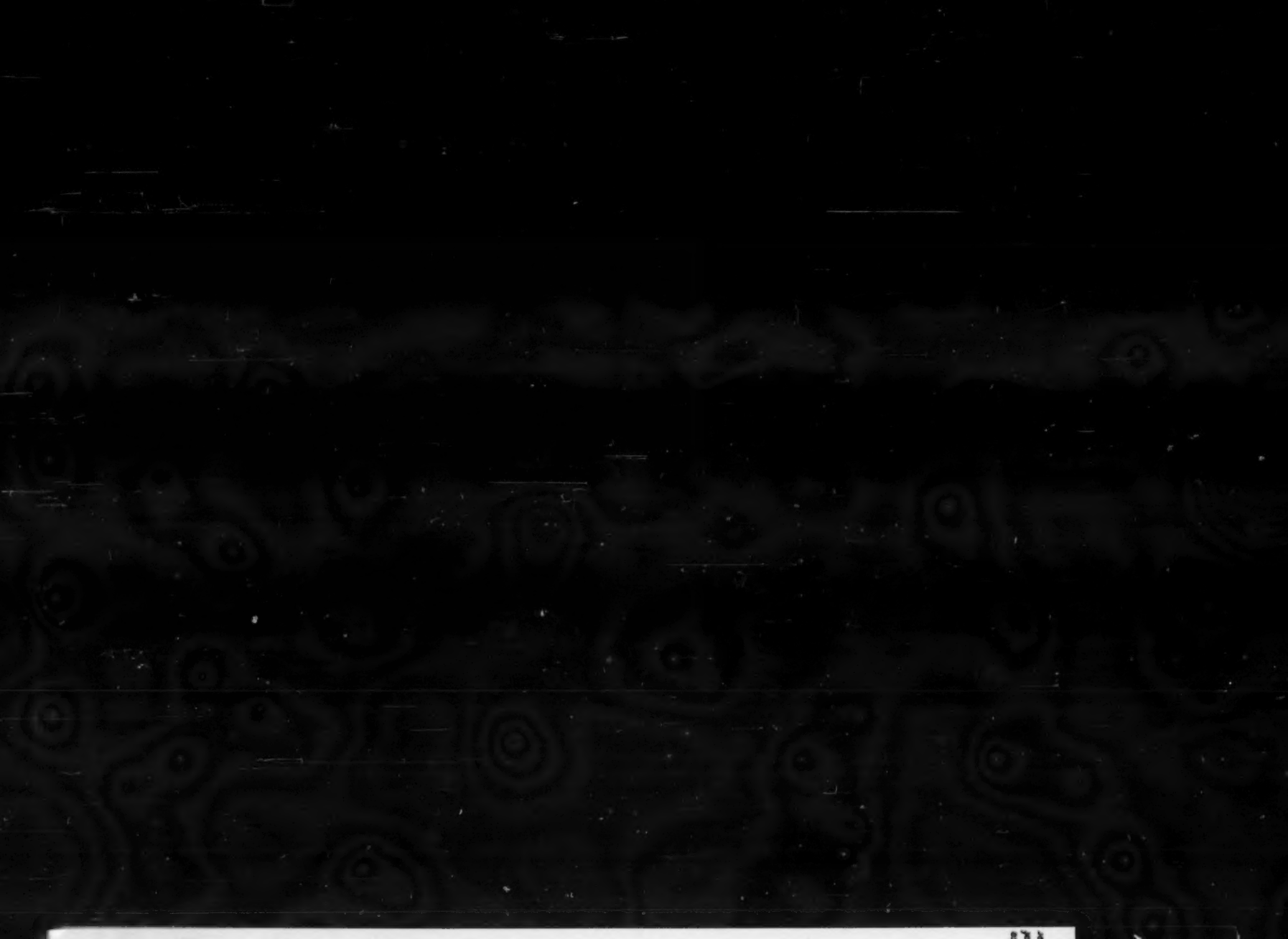
Branches: Cleveland, Ohio and Edgewater, N.J.